SEROLOGIC CLASSIFICATION OF SELECTED CASES OF MYELOMA

by

Lora Allred

A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Master of Science

Department of Microbiology

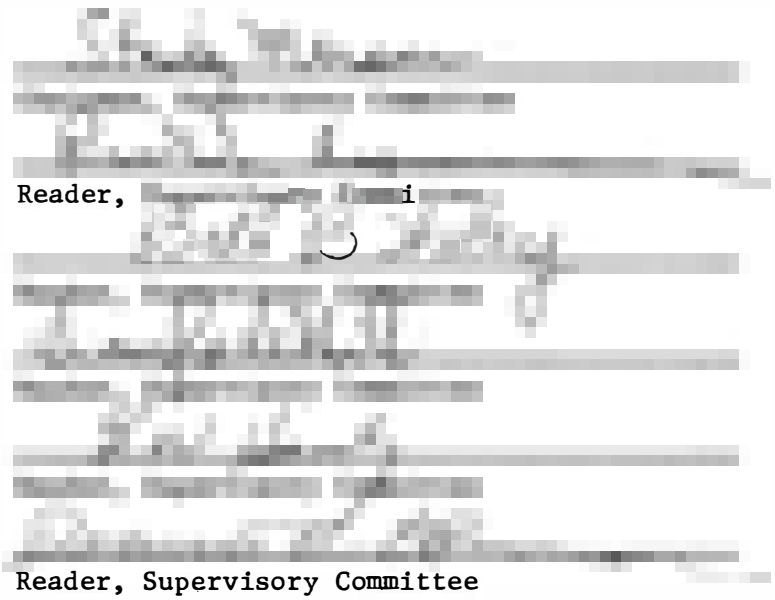
University of Utah
August, 1967

This Thesis for the

Master of Science Degree

by
Lora Allred

has been approved
July, 1967



Reader,

Reader, Supervisory Committee

Head, Major Department

Dean, Graduate School

The author wishes to express her appreciation to Dr. Stanley Marcus and Mr. J. LaMar Robbins for guidance and encouragement in the preparation of this thesis.

Grateful acknowledgement is made to Dr. Louis P. Gebhardt, Dr. Douglas W. Hill, Dr. Bill B. Wiley, Dr. DeWitt T. Hunter and Dr. Edward W. Hanly for their suggestions and criticisms.

She would also like to acknowledge the assistance of Mrs. Ann H. Thomas who proofread and typed this thesis.

Appreciation is expressed for the use of laboratory facilities and records of the Latter-Day Saints Hospital made available through the courtesy of Dr. John H. Carlquist and Dr. Gilbert A. Hill. Work described has been supported by the Latter-Day Saints Hospital Research Foundation and Department of Pathology, the University of Utah Medical Center, Department of Pathology, and the National Institute of Allergy and Infectious Diseases (CA 07302).

TABLE OF CONTENTS

ABSTRACT

Serologic and electrophoretic procedures were employed in the study of serum and urinary proteins from twenty-six patients with multiple myeloma. Hematological, radiological and other findings associated with myelomatous disease, or otherwise applicable to interpretative analyses of serological data, were reviewed. The serum and urinary proteins of these patients were found to have distinct electrophoretic properties and serologic characteristics. In this series, the M-components of four patients were classified serologically as Gamma-A paraproteins, two as Gamma-D paraproteins, fifteen as Gamma-G paraproteins and five were nonreactive with the available class specific antisera. Antigenic typing of twenty of these proteins with light chain antisera gave a 1:1 ratio; that is, ten proteins reacted with lambda type light chain antisera and ten proteins reacted with kappa type antisera.

Considerable diversification was present in the electrophoretic patterns of the serum and urinary proteins. The concentration of the electrophoretic fractions varied with the degree of proteinemia or proteinuria. Hyperproteinemia was evident in only fifty per cent of the gammopathies included in this study. The evidence of an abnormal component ranged from negligible to the appearance of three distinct peaks in an electrophoretic region where only one peak usually occurs. Quantitative estimation of an M component in the sera ranged from negligible to 8.8 gm/100 ml. Proteinuria was demonstrated in the urine of eighteen of the twenty-three patients tested.

The immunoglobulins were quantitated by a radial immunodiffusion technique. A correlation was apparent with the light chain antigenic type and alterations of the Gamma-G immunoglobulin level in the Gamma-A and Gamma-D myelomas. Myeloma proteins consisting of lambda type light chains were associated with reduced Gamma-G serum levels, whereas myeloma proteins consisting of kappa type light chains were associated with quantitatively normal serum Gamma-G levels.

TABLES AND FIGURES

TABLES

FIGURES

SEROLOGIC CLASSIFICATION OF SELECTED CASES OF MYELOMA

INTRODUCTION

Derangements in gamma globulin synthesis are known to occur in conjunction with myelomatous processes. These changes may be observed as a narrow, dense protein zone in the electropherogram, or as a flocculent precipitate in thermal solubility testing for Bence Jones proteins. However, if the presence of a monoclonal peak in zone electrophoresis and the thermal properties of the Bence Jones proteins were the sole criteria for distinguishing the paraproteins, a goodly share of the immunoglobulin aberrations would remain undetected. The development of immunochemical procedures has provided methods for the critical description of the immunoglobulins in the pathologic state.

The paraproteins produced in the plasma cell dyscrasias appear to be derivatives of one or more of the immunoglobulins found in normal sera and are distinguishable from the polypeptides of these globulins. The presence of each isolated antibody within the immunoglobulin classes of normal sera reflects an immune response of the body. This response is partially characterized by the production of a distinct antibody with well defined physicochemical properties. In a like manner, the protein dyscrasias are characterized by the elaboration of large quantities of a homogeneous protein with well defined physicochemical properties. The uniqueness of these properties may account, to a large extent, for the individual clinical and pathologic manifestations, as well as the characteristic therapeutic response seen in individual cases of multiple myeloma. Because there are many diversified ways in which a dysproteinemia may be expressed, clinical

management becomes difficult unless classification of the abnormal protein into one of the constituent groups of immunoglobulins with related properties can be made.

A definitive classification of the gammopathies depends upon physicochemical and serologic characterization of the serum proteins, an examination of the bone marrow, radiologic bone surveys and clinical evidence of the disease. The low incidence of the gammopathies in the population makes it difficult to obtain a significant number of cases for evaluation. Nonetheless, continual studies must be conducted and evaluated on the limited number of cases encountered if a working knowledge of the myelomatous processes is to be obtained. Therefore, appropriate physicochemical and immunochemical procedures have been performed on specimens from selected myeloma patients. The results of these studies, together with additional findings, are presented herein.

REVIEW OF LITERATURE

I. HISTORICAL DEVELOPMENT OF THE IMMUNOGLOBULINS

For many years antibody formation remained an obscure phenomenon related in some manner to the properties of the serum. The contributions of investigators toward the elucidation of the biophysical and biochemical properties of antibody molecules has spanned over a century, and still many of the facets of this problem remain unresolved. However, today, antibody formation has been established as a function of globulin synthesis (Janeway and Gitlin, 1957; Dreyer and Bennett, 1965).

The characteristic solubility properties of the plasma globulins was first noted by the French Physician Prosper-Sylvain Denis prior to 1859.(Denis, 1859). This observation led to the demonstration of differences in solubility of protein molecules when exposed to aqueous solutions of neutral salts, i.e. ammonium sulfate (Pohl, 1886), sodium sulfate (Howe, 1921a, 1921b), sodium sulfite, magnesium sulfate (Hammarsten, 1897), potassium citrate (Jameson, 1937), and mixtures of phosphates (Butler and Montgomery, 1933). These reactions are dependent upon many factors, namely, the concentration of the salt, the pH and the temperature. The exact chemical interaction involved in producing this effect is still incompletely understood. It is thought that the ionic groups on the surface of the protein molecules compete with salts for the molecules of solvent. This competition produces a shift in the equilibrium causing the proteins to be precipitated as a result of the salt ions being bound to the available aqueous phase (Cohn and Edsall, 1943; Haurowitz, 1935, 1936a, 1936b).

These salting-out procedures demonstrated that more than one separate protein entity, i.e. albumin and globulin, did in fact reside within the serum. During this same period, it was recognized that the globulin class could be further subdivided on the basis of solubility characteristics in salt-free water. The soluble globulin fraction was referred to as pseudoglobulin, while the precipitable portion was called euglobulin (Burckhardt, 1883; Marcus, 1899; Porges and Spiro, 1902; Hardy, 1905).

It was not until the classical moving boundary electrophoretic studies of Tiselius (1930) that the distinct heterogeneity of the serum proteins was firmly established. This study prompted an improved electrophoretic system which characterized the serum globulin fraction as being a mixture of at least three components. Separation occurred as a function of the unique isoelectric points of each of these components when subjected to an electrical field at a known pH. The characteristic globulin fractions obtained were designated alpha, beta and gamma in order of decreasing mobility (Tiselius, 1937b). Of particular interest was the finding that pseudoglobulin was not confined to the albumin fraction, but contained alpha globulin and a small percentage of gamma globulin, while euglobulin consisted largely of beta and gamma with less alpha (Tiselius, 1937a, 1937b). At this early date, the marked possibility of further heterogeneity within each of the electrophoretically separable fractions was recognized.

Immediately succeeding the identification of the alpha, beta and gamma globulin serum components, Tiselius noted the correlation of

antibody activity in purified preparations with the gamma globulin migratory fraction (Tiselius, 1937c). Currently, however, there is considerable evidence to indicate that it is erroneous to restrict the classification of antibodies to this electrophoretic fraction. Antibodies with different specificities, or even antibodies which cross-react may have electrophoretic mobilities ranging from the alpha through the gamma region (Williams and Grabar, 1955). Indeed, further studies in the 1940's with antibody-rich protein fractions led to the discovery of an antibody component with an electrophoretic mobility intermediate to gamma globulin and beta globulin, which was accordingly designated T, $beta_2$ or $gamma_1$ by various investigators (van der Scheer, et al., 1940; Kekwick and Record, 1941; Deutsch and Nichol, 1948).

A description of $beta_{2A}$ globulin did not ensue until the introduction of the immunoelectrophoretic technique of Grabar and Williams (1955). This technique utilized the electrophoretic mobilities of proteins in agar gel in combination with the immunologic specificities obtained from the gel diffusion technique of Oudin (1946). The resultant precipitin arcs have enabled the detection and semi-quantitation of more than 30 separate serum components (Crowle, 1961).

Heremans (1960) introduced the term "immunoglobulins" to include the $gamma_2$, $gamma_{1M}$ and $beta_{2A}$ proteins. To date, there are three major (IgG, IgA, IgM) and two minor (IgD, IgE) distinct structural classes of immunoglobulins that have been reported (Rowe and Fahey, 1965b; Ishizaka et al., 1966). The designation "immunoglobulin" is

used broadly to distinguish the production of antibodies by an immune mechanism from the globulin produced "normally", or at least from an unknown antigen source. This latter non-specific antibody has been ascribed to a low combining strength of antibody with the antigen (Haurowitz, 1942), an anamnestic response to a previously exposed antigen (Boyd and Bernard, 1937), or to a nonantibody function of a certain portion of the "natural" gamma globulin (Larson and Tomlinson, 1952; Lawson et al., 1955). As the sensitivity of the methods for detecting antibody reactions with the corresponding antigen increase, these "inert" gamma globulins may be identified as antibodies. The definition of the immunoglobulins has also been extended to include the myeloma proteins, Bence Jones proteins, and certain normal urinary protein components (Fahey, 1965). It appears that these latter proteins do not function as antibodies (Gutman, 1948; Fahey, 1965), but are sufficiently related structurally to merit inclusion in the immunoglobulin classification. In the instance of the myeloma protein, Kunkel and Slater (Kunkel and Slater, 1952; Slater et al., 1955) demonstrated a precipitin reaction against the antisera of normal human gamma globulin. Bence Jones proteins (Mannik and Kunkel, 1962; Fahey and Solomon, 1963; Nachman, et al., 1965) and normal urinary proteins (Webb, et al., 1958; Franklin, 1959; Remington, et al., 1962; Stevenson, 1960; Berggard, 1961) which exhibit a serologic and structural relation to the immunoglobulins have also been described.

Due to the multiplicity of investigators and the different animal species used in the study of the structure and function of antibodies,

a formidable terminology has emerged to describe the antibody molecules. The nomenclature recommended by the World Health Organization (1964) and some of the properties which characterize the immunoglobulins are given in Table 1.

Table 2 gives the products obtained from enzymatic and reductive cleavage of the antibody molecule by various investigators with the accepted W.H.O. nomenclature, the synonomous terminology used by the investigator, and the species used to obtain the product.

II ENZYMATIC AND REDUCTIVE CLEAVAGE

In the past, the antibody molecule was thought to consist of a single polypeptide chain (Porter, 1950). When the presence of more than one N-terminal amino acid in myeloma proteins was observed a multichain structure was suspected (Putnam, 1955). Because of the relative homogeneity and the copious production in disease states, the myeloma and Bence Jones proteins have served as model globulins to help determine the conformation of the antibody molecule. However, a rigorous classification of the subunit structure was not appreciated until enzymatic and reductive cleavage were applied to the antibody molecule (see Table 2).

Enzymatic proteolysis has been used extensively in characterizing native proteins. In order to derive an understanding of the mechanisms of enzyme hydrolysis, Tiselius and Eriksson-Quensel (1939) subjected ovalbumin to pepsin and studied the enzymatic degradation process in detail. Proteolysis was first applied to the gamma

TABLE 1

HUMAN SERUM IMMUNOGLOBULINS

IMMUNOGLOBULIN W.H.O. NOTATION	SYNONYMS	MOLECULAR WEIGHT	SEDIMENTATION COEFFICIENT	ELECTROPHORETIC MIGRATION	NORMAL SERUM LEVEL (mg/ml)
γG or IgG	γ, 7S$_\gamma$ 6.6S, γ_2 γ_{ss}	150,000	6.6S	gamma	12.4 ($\pm$2.2)
γA or IgA	B_2A, γ_1A	150,000	6.6S (9,11,13S)	slow beta	3.9 ($\pm$0.9)
γM or IgM	γ_1M, B_2M 19S, $\bar{=}$ macroglobulin	900,000	18S (24S, 32S)	between gamma and beta	1.2 ($\pm$0.35)
γD or IgD	γ_1 J	160,000	7.04S	between gamma and beta	0.03 (0.-0.4)
γE or IgE	None		8S	between gamma and beta	unknown (<0.03)

TABLE 2

DEGRADATION OF THE GAMMA GLOBULIN MOLECULE

DEGRADATION METHOD	ANTIBODY SUBUNIT W.H.O. NOTATION	INVESTIGATOR'S NOTATION	SPECIMEN	REFERENCE
Enzyme Digestion				
1. Papain Fragments	Fab-fragment (antigen-binding)	I, II	Rabbit serum	Porter (1959)
		A, C	Cohn Fraction II Human γ-globulin	Franklin (1960)
		S (slow)	Human γ-globulin	Edelman et al., (1960)
	Fc-fragment (crystallizable)	III	Rabbit serum	Porter (1959)
		B	Cohn Fraction II Human γ-globulin	Franklin (1960)
		F(fast)	Human γ-globulin	Edelman et al., (1960)
2. Peptic Fragments	$F(ab')_2$ fragment	5S divalent fragment	Rabbit antibody	Nisonoff et al., (1961)
Enzyme Digestion & Reduction				
1. Papain Digest	Fd-fragment	A piece	Fab-fragment	Frangione & Franklin (1965a)
2. Peptic Digest	Fab'	Univalent fragment	$F(ab')_2$ fragment	Nisonoff et al., (1961)
Reduction	Heavy chain	A	Rabbit antibody	Porter (1962)
		H	7S γ-globulin Myeloma globulin Bence Jones protein	Edelman & Benacerraf (1962)
	Light chain	B	Rabbit antibody	Porter (1962)
		L	7S γ-globulin Myeloma globulin Bence Jones protein	Edelman & Benacerraf (1962)

globulin molecule when Pope (1939) examined the effect of a pepsin digest on horse diphtheria antitoxin, but the major contribution eliciting an understanding of the structure of the antibody molecule can be attributed to the work of Porter (1959) with papain. The thoroughness of this work, coupled with that of Fleischman (1963), set a pattern for the investigation of the immunoglobulins.

Porter (1959) observed that when pooled rabbit gamma globulin was partially hydrolyzed by crystalline papain, the antibody-combining site was retained in two of the three resulting fragments. These fractions were designated I, II and III in order of elution from a carboxymethyl cellulose column. Ultracentrifuge studies of these three components revealed a single peak at S_{20w}, 3.5, suggesting that the 6.6S gamma globulin molecule has been split into three fragments of similar size. Palmer et al., (1962) have subsequently reported that the differences obtained in fractions I and II probably reflect the heterogeneity of the antibody population within a pooled preparation, rather than reflecting a difference in the subunits within the same molecule.

A number of workers adapted the experimental approach of Porter's work to an investigation of the cleavage of human gamma globulin (Edelman et al., 1960; Franklin, 1960; Stiehm et al., 1960; Hsiao and Putnam, 1961). Three major components were also obtained when Cohn fraction II of human gamma globulin was dissociated with papain and cysteine (Franklin, 1960). Two of these subunits, A and C, contained a univalent antibody site, while the third piece, B, had no

antibody site, differed in physical properties and could be crystallized. A further clarification of the role of enzymatic degradation was made possible by utilizing immunoelectrophoresis in conjunction with papain hydrolysis of the human gamma globulin molecule(Edelman et al.,1960). In this instance, the two components that were obtained from the digest were compared electrophoretically and immunoelectrophoretically with the untreated purified gamma globulin molecule. One of these fractions migrated faster (F) than the undigested molecule and the other was electrophoretically slower (S). Immunoelectrophoretically, two precipitin arcs were demonstrated with the original 7S molecule. The S fraction, which was present in about twice the concentration of the F fraction, was antigenically related to the inner arc, while the F fraction was identified with the outer arc.

Papain cleavage of the antibody molecule is effected by the hydrolysis of peptide bonds formed by the carboxyl groups of alpha-amino substituted arginine and lysine, and to a lesser extent derivatives of glutamine, histidine, glutamic acid, leucine, glycine, and tyrosine. The magnitude of this hydrolysis is dependent upon the availability of reactive groups according to the conformational structure of the molecule (Hill, 1965), and upon the concentration of the protein, reducing agents, papain, and urea used in the degradation process (Gyenes et al.,1966). It has been demonstrated electrophoretically that spontaneous degradation of stored human gamma globulin produces products similar to those obtained by enzymatic

digestion with papain, trypsin or plasmin (Skvaril, 1960; Augustin and Hayward, 1960).

A second approach to the degradation of the antibody molecule, reductive cleavage, has been more productive in yielding information for structural analysis. Reductive cleavage employs the disruption of disulfide bonds by a variety of agents such as mercaptoethanol, or other thiols, followed by stabilization of the reoxidizable sulfhydryl groups by alkylating agents, urea or acids. A subsequent "clean" separation of the fragments is obtained with ion exchange resins, differential dialysis, gel filtration or electrophoresis (Fraenkel-Conrat, 1963).

In 1959, Edelman showed that the human 7S molecule, when exposed to mercaptoethanol in 6 M urea, was disaggregated from a molecular weight of about 150,000 to a molecular weight of 50,000-60,000. This decrease indicated that the antibody molecule was probably composed of more than one peptide chain held together by interchain disulfide bonds. The products obtained by this reduction were biologically inactive and were insoluble in most aqueous solutions (Edelman and Poulik, 1961). Further studies with globulins of other species (Edelman and Poulik, 1961; Franek, 1961; Ramel et al., 1961) confirmed these original findings, thus demonstrating a similarity in general structure, i.e. more than one peptide chain per molecule. Previous studies relating to the multiplicity of the antibody peptide structure relied heavily on N-terminal group determinations. However, these amino acid analyses of gamma globulins from different species do not

correlate with the above results (Porter, 1960), which would suggest that some of these end groups are substituted or rendered inactive (Edelman and Poulik, 1961).

Since the retention of biologic activity in the degraded molecule is essential in determining the relationship of antibody function to antibody structure, a less drastic procedure that would not result in denaturation was sought. Under conditions of mild reduction, with mercaptoethanol in a neutral aqueous solution followed by fractionation in acid solution, this criterion was satisfied (Porter, 1962; Fleischman, et al., 1962). With this method the gamma globulin molecule was split into two types of polypeptide chains. The major component was designated A chain and the second fraction, B chain. The results of this study led to the postulation of a 4-chain antibody structure (see Figure 1) (Porter, 1962). An analysis of amino acid composition, molecular weight determinations and of the products obtained under mild and full reduction was consistent with this hypothesis (Fleischman, et al., 1963).

A second linear representation of the antibody molecule was later proposed by Tanford (Noelken et al., 1965) to account for the products obtained upon exposure to proteolytic enzymes and reducing agents (see Figure 2). The experimental work with proteolysis and reductive cleavage methods have shown that the antibody molecule is composed of at least two types of polypeptide chains. These polypeptides have been designated "heavy" (H-chain) and "light" (L-chain) on the basis of molecular weight determination (see Table 2 and Figure 3). Heavy

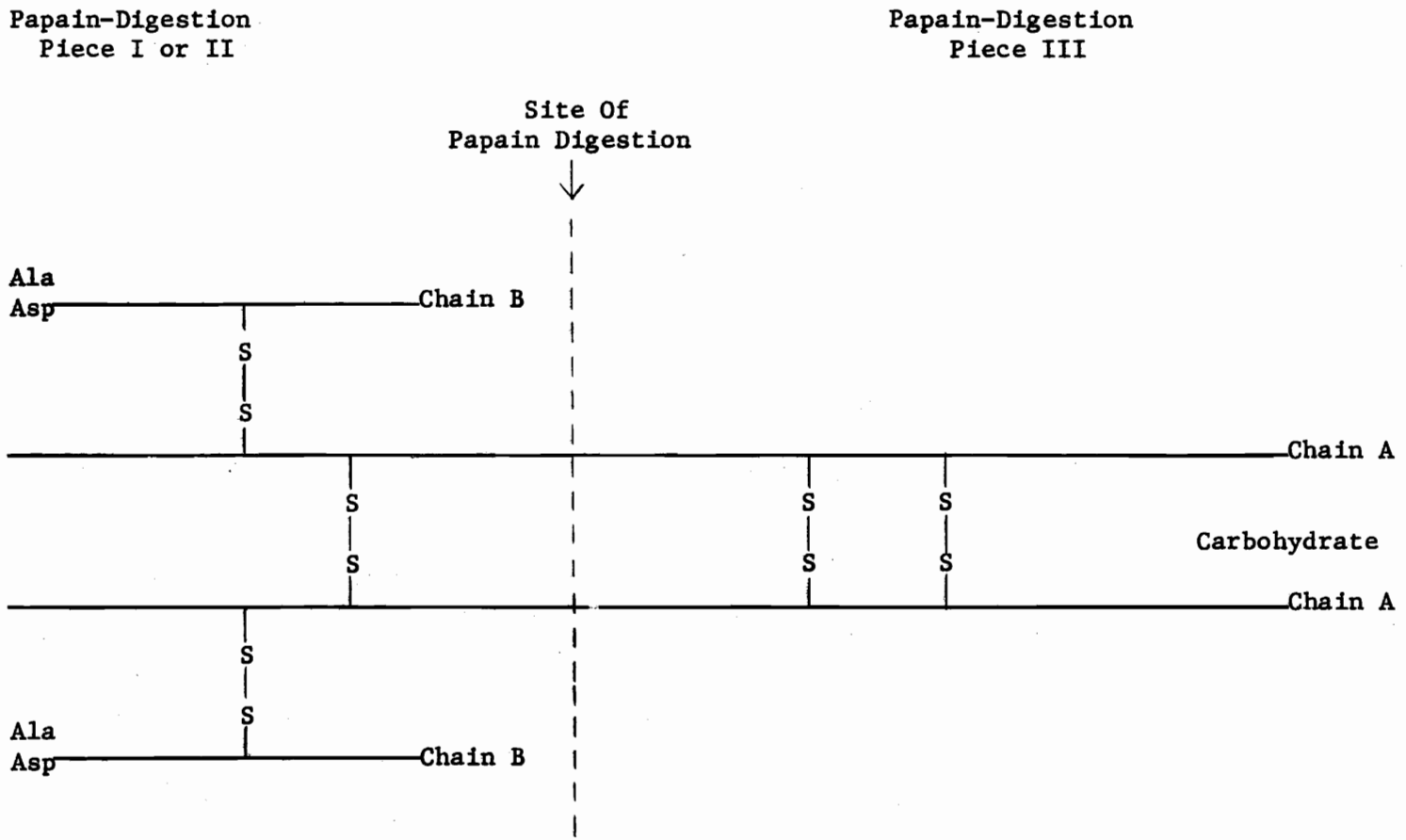


Figure 1. Diagrammatic structure of rabbit gamma globulin (Porter, 1962)

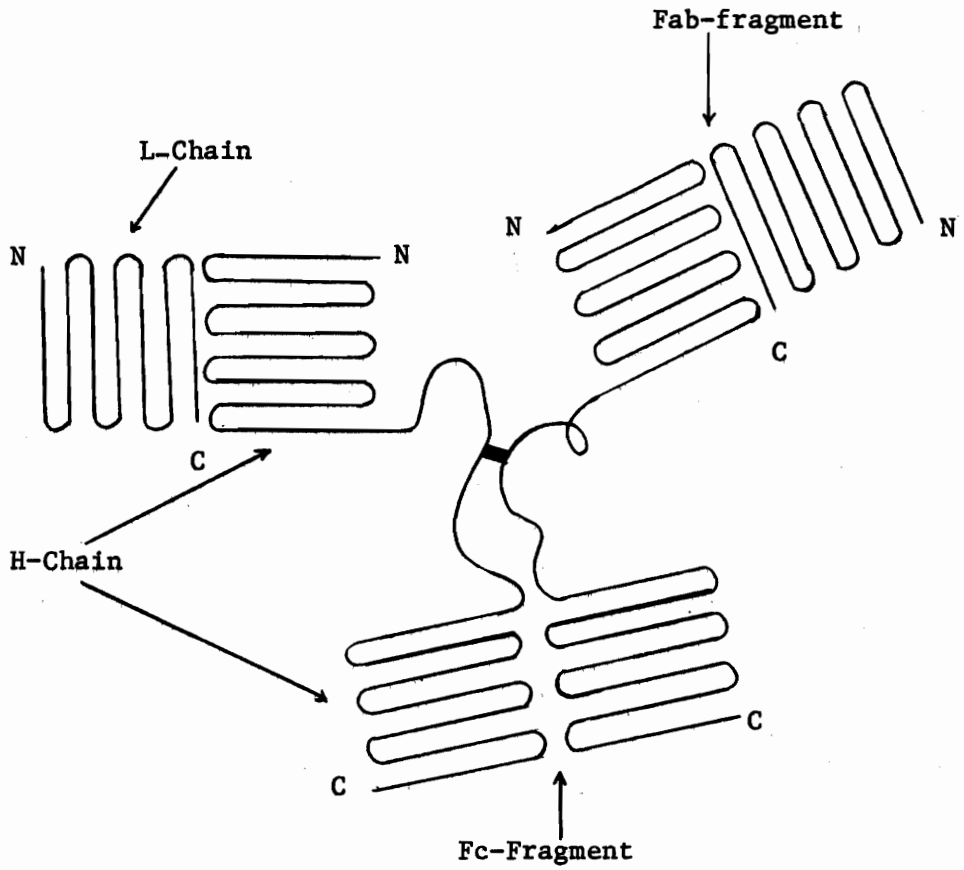


Figure 2. Schematic Representation of a Y-Globulin Molecule as Proposed by Tanford.

The folded portions of the polypeptide chains represent globular oriented portions of Y-globulin. The random coil in the central portion of the Y-globulin molecule carries the disulfide bond. It represents the portion of the molecule most susceptible to enzymatic digestion (Merler and Rosen, 1966).

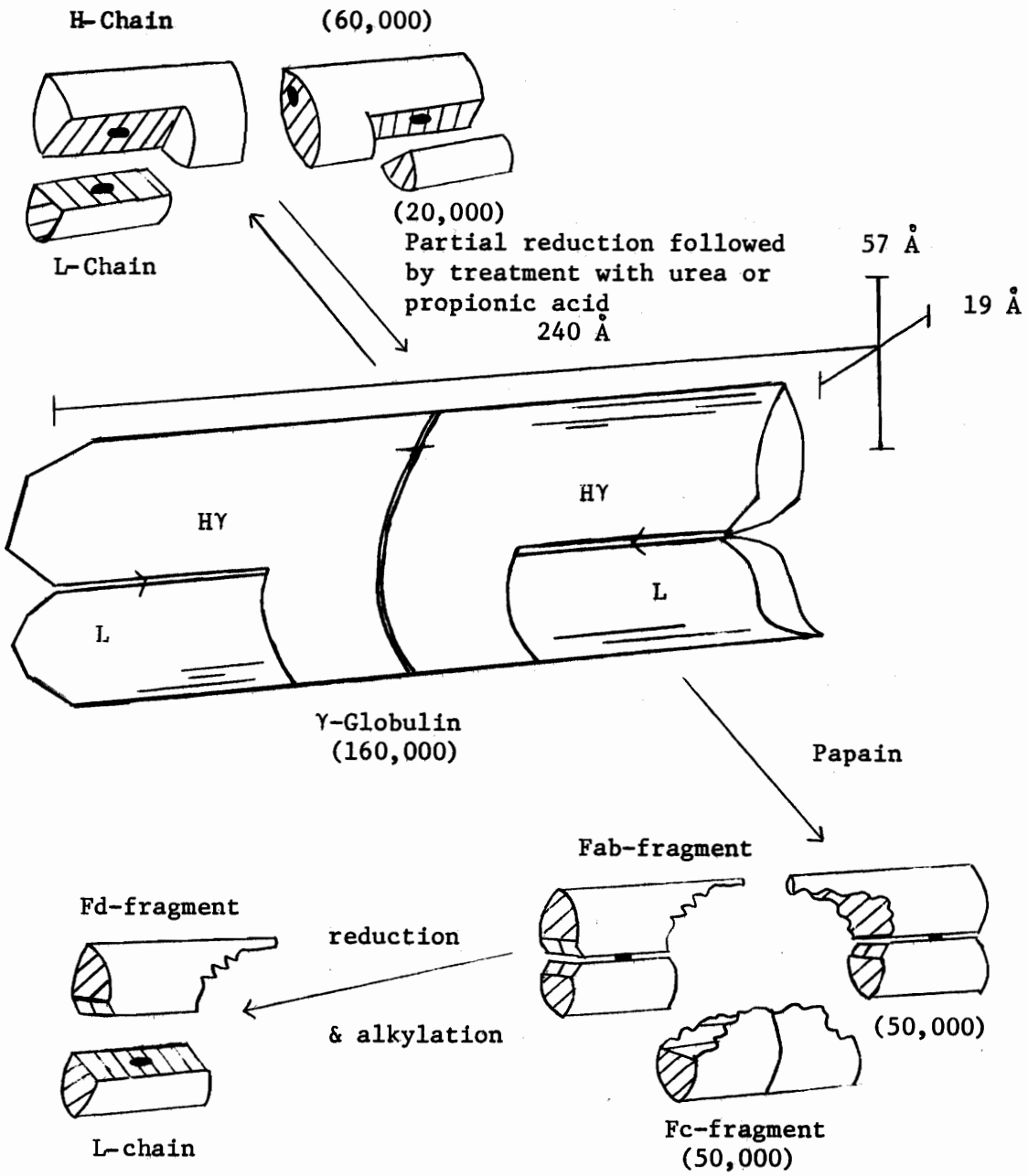


Figure 3. Schematic diagram illustrating enzymatic digestion and partial reduction of the 7S antibody molecule. Numbers in parentheses are approximate molecular weights. (Adapted from Edelman and Gally, 1964)

chain bonding in the antibody molecule is now thought to be effected by a single disulfide bond rather than by the three inter-H-chain disulfide bonds originally proposed by Porter (see Figure 1). This second model allows for the independent rotation of each of the Fab-fragments and the Fc-fragment around this inter-H-chain bond (see Figure 2). That such rotation does occur is supported by measurements of the depolarization of fluorescence energy (Noelken et al., 1965).

A comparative analysis of the reductive products obtained in the presence and absence of denaturing agents was obtained by starch gel electrophoretic characterization in 9 M urea (Porter, 1962; Cohen, 1963a, 1963b). Migration patterns revealed that the H-chain was equivalent to the A fraction, and that the B component could be equated with the L-chain. It has been reported that loss of immunologic activity of the antibody molecule with extensive reduction is due to the cleavage of 12 to 18 out of the 20 disulfide bonds, while cleavage of a maximum of 5 disulfide bonds with gentle reduction retains this activity (Porter, 1962; Fleischman, et al., 1962).

The correlation of Fab and Fc (see Table 2) fragments with the heavy and light polypeptide chains was demonstrated by a number of investigators through immunologic specificities (Fleischman et al., 1962; Olins and Edelman, 1962; Cohen, 1963a, 1963b; Nussenzweig, et al., 1964), molecular weight determinations (Marler et al., 1964), and radioisotope labelling (Olins and Edelman, 1964). From these analyses, it would appear that papain digestion produces a crystallizable Fc fragment devoid of antibody activity which comprises a

portion of the H-chain, and two Fab-fragments each consisting of the remaining portion of heavy chain and the entire L-chain. Each of the Fab-fragments contain a single antigen binding site. Subjection of the Fab-fragment to reduction and aklylation causes the heavy chain portion to dissociate from the L-chain (Fleischman, et al., 1963; Fougereau and Edelman, 1965; Heimer, 1966). This N-terminal H-chain piece is separable from the light chain by gel filtration and has been designated "Fd fragment" (see Figure 3).

A topographical model of the 7S antibody molecule, which takes into account all the known products obtained by degradation with proteolytic enzymes and by reduction of the interchain disulfide bonds, is presented in Figure 3 (Edelman and Gally, 1964). Edelman and Gally viewed the antibody molecule as a cylinder of elliptical cross section with dimensions of 240°A in length by 19°A in width, consisting of a symmetric arrangement of two light polypeptide chains with molecular weights of 20,000-24,000 and two heavy chains with a molecular weight of 55,000-60,000. Polypeptide pairing is accomplished by the presence of three interchain disulfide bonds.

Recently it has been suggested, on the basis of data obtained by electron microscopy, that the correct configuration of the gamma immunoglobulin may be trianguloid, 80°A wide at the base, 35°A high and 105 to 120°A in length (Feinstein and Rowe, 1965).

III ANTIBODY SPECIFICITY AND THE STRUCTURAL RELATIONSHIP

The molecular localization of an antigen combining site on each

of the two identical Fab antibody fragments supports the concept that the 7S antibody molecule is bivalent (Porter, 1959; Kunkel, et al., 1963). Further evidence of this valency is obtained under conditions of maximum antigen-antibody complexing in the presence of excess antigen, by the utilization of ultracentrifugal, electrophoretic and radioisotopic analyses, and equilibrium dialysis studies (Porter, 1960). The identification of two combining sites of the same antibody molecule has led to an investigation of the possibility of diverse biologic specificities at each of these two sites. It has been found that heteroligating antibodies can be produced by the formation of an artificial antibody from two monovalent fragments with different specificities (Nisonoff and Rivers, 1961). However, all analyses of the 7S molecule produced physiologically reveal that the two combining sites have the same specificity (Singer, 1965).

Attempts to assign the binding site solely to either the L-chain or the Fd-fragment have been inconclusive. Recombination studies with separated specific and non-specific heavy and light chain polypeptides have failed to determine if the specificity of antigen binding activity resides exclusively in one of the polypeptide units. Specificity may reside either with the H-chain or with the L-chain, and in certain instances both chains are implicated (Grey, et al., 1965; Lamm et al., 1966). The observable variability in antibody behavior appears to be dependent upon the particular antibody involved, i.e. any given antibody seems to be characterized by a consistent localization of the antigen combining site.

A definition of the size of the antigen combining site rests upon inference from non-specific evidence. It has been observed, in certain instances, that an antigenic stimulus may produce a population of antibody molecules with site dimensions of variable size. This is manifest in studies with the antidextran and anti-DNP (dinitrophenyl) antibodies (Kabat, 1957; Singer and Doolittle, 1966). On the basis of information determined from free energies of antibody-hapten reactions by Schlossman and Kabat (1962), Merler and Rosen (1966) have estimated the area of the combining site to be no more than 5% of the entire molecule. In terms of amino acid composition, the region of antibody-antigen interaction has been estimated to consist of no more than ten to twenty amino acid residues (Karusch, 1962).

It has been proposed that the diverse biologic specificity found within the population of antibody molecules might be attributable to variable stereochemical orientation of the molecule at the active site, as a direct or indirect result of interchain and intrachain non-covalent or disulfide bonding. The structural features of the antibody molecule that could contribute to the three-dimensional configuration are 1) the sequence of amino acid residues within each polypeptide chain, 2) the bonding of the polypeptide backbone, and 3) the conformation produced from the interaction of the four polypeptide chains. Each of these suggestions has been examined either separately or integrally to determine their contributing role in determining antigenic specificity.

Early work with antibodies led to the hypothesis that amino acid

composition and sequence were identical for antibodies of different specificities, but that complementary antigen-antibody binding could be produced by a localization of different portions of the chain at the active site. This suggestion could be explained if there was a multiplicity of ways in which the chain could be folded. Specific folding was thought to be mediated by the formation of variable secondary and tertiary structures resulting from noncovalent or disulfide bond stabilization rather than to fluctuation in the amino acid sequence (Karusch, 1957). More recently, studies with tryptic peptides and reoxidized polypeptide chains (Fleischman 1966) place some doubt upon certain aspects of this hypothesis.

In order to examine the role of primary structure in regulating the specific folding of antibody chains, noncovalent and disulfide bonds have been cleaved and specific activity reevaluated after the molecule was refolded (see Figure 4, Watson, 1965). Specific activity was recovered with the disruption in quanidine of only noncovalent bonds (Buckley, et al., 1963; Noelken and Tanford, 1964). In as much as the formation of an active configuration could still be directed by disulfide bonds, methods for disruption of both non-covalent and disulfide bonds were also employed (Haber, 1964; Whitney and Tanford, 1965). After exposure of the native protein to reducing conditions, the chains were found, by optical rotation, to be completely unfolded. The binding of the reoxidized and refolded soluble portion of the specific protein was compared to a control of nonspecific antibody. The nonspecific fragment showed no activity,

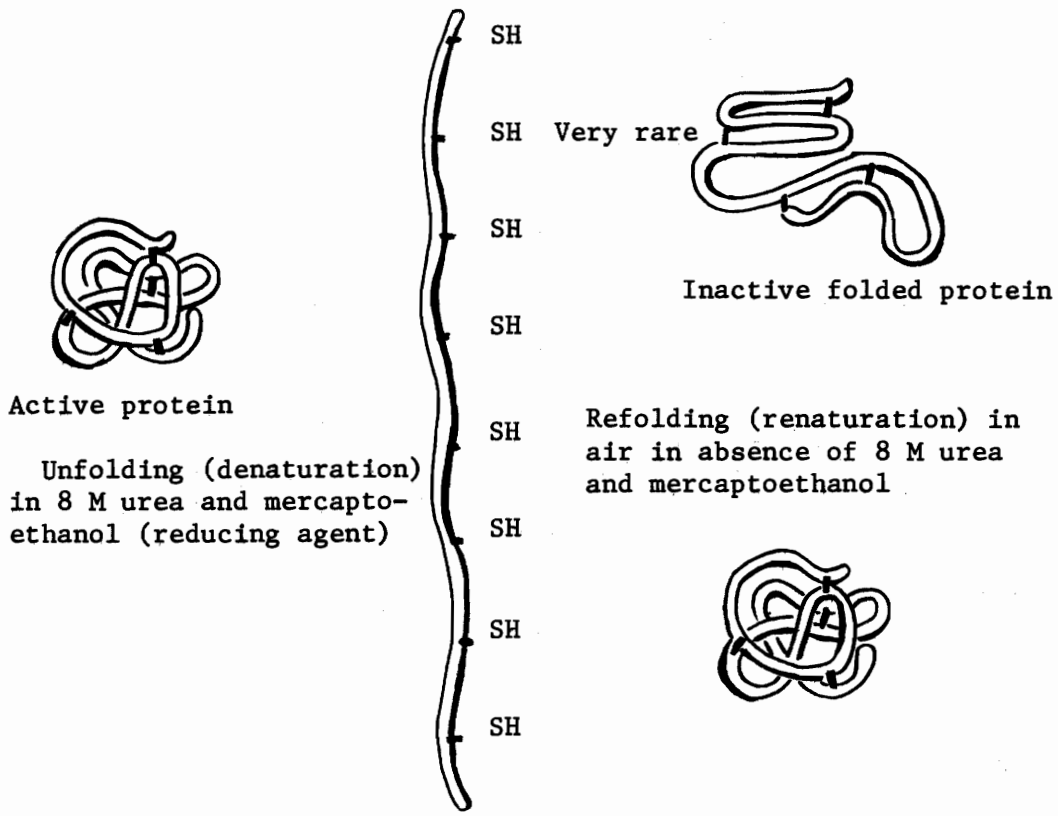


Figure 4. Schematic illustration of the fate of S-S bonds during protein denaturation and renaturation. When the denaturing agents are removed, most of the polypeptide chains resume the native configurations with the original S-S bonds. Only a few polypeptide chains old up in an inactive form characterized by a different set of S-S bonds than those found in the native molecules. (Watson, 1965)

whereas 20 per cent of the specific material regained binding activity. These studies tend to demonstrate that the role of disulfide and noncovalent bonding as a determinant in the peculiar folding of the chains of the antibody molecule must be secondary to the amino acid sequence. If this is the case, then the extensive heterogeneity of the immunoglobulins must be sought through an explanation due to amino acid differences.

There is no detectable significant difference in assays for relative amino acid content within each subgroup of the immunoglobulins (Gitlin and Merler, 1961; Koshland and Englberger, 1963), but there is a unique primary sequence of amino acid residues associated with both the H and L polypeptide chains. Contiguous to the variant region of the protein is an invariant amino acid sequence common to the particular chains. Peptide analyses indicate that the distinctive amino acid portion appears at the N-terminal end of the light chain, while the amino acid sequence on the C-terminal end of this polypeptide remains constant. Marked similarities of the heavy chains are seen on peptide maps of the Fc-fragment. The slight differences observed in a few of these H-chain peptides do not appear to reflect diverse antigenic specificities, but represent the variations characteristic of the genetic subgroups, We, Ne, Vi and Ge (Kunkel, et al., 1966; Merler and Rosen, 1966; Milstein, 1964; Titani and Putnam, 1965; Hilschmann and Craig, 1965). Due to impediments in preparing the Fd-fragment, sequence differences on this half of the H polypeptide are derived by inference from studies

of the entire heavy chain and the Fc-fragment (Frangione and Franklin, 1965a and 1965b). These studies would indicate that considerable heterogeneity in amino acid sequence also exists within this, the amino end, portion of the molecule.

In addition to the role assigned to the primary structure of antibody in determining biological specificity, another structural feature has been indicated that imparts molecular specificity. Since the immunoglobulins consist of more than one peptide chain, the interaction of tertiary and quanternary structures due to cross linkages of the disulfide bond must be accounted for. Although there is no confirmatory evidence that would ascribe specificity of the antibody site to tertiary structure due to random disulfide bonding, cross-reactions with the human immunoglobulins G and M due to tertiary and quanternary structure have been reported (Seligmann, et al., 1966). The possibility of this precipitin reaction being due to the common structural amino acid sequence present at the carboxy-terminal portion of the H-chain is made unlikely by the confinement of reactivity to the Fab-fragment. Cross-reactivity due to identical primary structure of the L-chains is ruled out by the use of two distinct antigenic determinants, type K and type L (see Section IV). Common antigenic determinants can be demonstrated in the IgM and IgG globulins. Conformational structure may account for this common denominator. Evidence for this likelihood is the necessity of heavy and light chain interaction before cross-reactivity can be observed.

IV IMMUNOGLOBULIN SUBUNIT CLASSIFICATION

As previously stated, most antibody molecules consist of four polypeptide chains, two identical L-chains and two identical H-chains, symmetrically joined by three disulfide bonds into an elliptical configuration. The heavy chains impart the specific characteristics for each class, whereas the light chains account for the properties common to all classes (Franklin and Stanworth, 1961; Olins and Edelman, 1962). The immunoglobulins have been categorized into major and minor classes, subclasses and genetic subgroups on the basis of antigenic differences within the polypeptide chains (Kunkel, et al., 1966; Fleischman, 1966). These differences are, in part, apparently a reflection of amino acid sequence differences (Singer and Doolittle, 1966), which appear to be independent of the primary structural variations present at the antibody combining site.

Because of the relatively low yield of immune globulins obtained from sera of normal individuals, for the most part, an identification of different antigenic determinants has depended upon the utilization of paraproteins from malignant disturbances. Each of the immunoglobulin molecules have been found to exist in elevated serum concentration concurrently with plasma cell dyscrasias, that is, conditions where there is an infiltration of abnormal plasma or lymphoid cells in the bone marrow or tissues in association with derangements of gamma globulin synthesis. In the instance of the immune globulin D, it is only because of proliferative changes that

the identification and description of the globulin itself has ensued (Rowe and Fahey, 1965a). However, protein descriptions based on reactions of identity or nonidentity with antisera from protein dyscrasias introduce certain advantages as well as certain inherent errors in classification.

First, globulins used as antigens from the plasmacytoid and lymphocytoid dyscrasias elicit the production of highly specific antibodies, characterized by sharp electrophoretic banding, precise mobility, and a discrete reaction of identity with the neoplastic antigen. However, several groups of investigators have reported that in the majority of instances, specific individual antibody reactivity may be absorbed out by repeated exposure to pooled normal gamma globulin (Grey, et al., 1965; Seligmann, 1965). These findings may indicate that the presence of large quantities of distinct homogeneous protein components simply represents some unknown highly selective mechanism for the production of a finite portion of the normal antibody spectrum, or it may be that the abnormal globulin is so closely related to the antibodies within the normal spectrum that cross-reaction is possible. While cross reactions with antibody from malignant clones allow for specific identification, it also limits classification to those proteins which are readily accessible because of a defective control in protein synthesis. In all probability many more classes, subclasses and subgroups will remain unidentified until selected by protein disturbances or until other methods for detection are utilized.

A second concern in the classification of the immunoglobulins is the differentiation of the H-chain subclasses from the major and minor immunoglobulin classes. In both cases, the antigenic determinants are present on the heavy chain polypeptide. If the cross reaction is confined to the Fc-portion of the heavy chain, then the protein is designated as a subclass. If no reaction occurs with the H-chain, a new immunoglobulin class is recognized. Recently, the validity of this approach has been questioned (Kunkel, et al., 1966), in that, partial cross reactivity of the different immunoglobulin H-chains has been reported (Kunkel, et al., 1965).

Using specific rabbit and monkey antisera, at least four subclasses have been delineated in the Gamma-G class (Dray, 1960; Kunkel, et al., 1964b; Terry and Fahey, 1964). These subclasses have been designated Ne (γ_{2a}), We (γ_{2b}), Vi (γ_{2c}), and Ge (γ_{2d}) (Kunkel, et al., 1964b: Martensson and Kunkel, 1965; Grey and Kunkel, 1964). Of the three antigenic determinants detectable by rabbit antisera (Fleischman, 1966), two have been found on the Fc-fragment (Terry and Fahey, 1964; Grey and Kunkel, 1964), while a third was detected on the $F(ab')_2$ fragment (see Table 2). The specificity was lost with the reduction of the latter fragment, indicating that the disulfide bond may be involved (Grey and Kunkel, 1964). The other classes of human heavy chain immunoglobulin components are less well studied.

Two different types of L-chains, kappa and lambda, are recognized serologically. Both of these antigenic types occur in the light

chains of all four of the immunoglobulin classes (Fahey, 1963). By utilizing the unique serologic reactivity of the M-proteins (see Section VI) antigenic subtypes within both the type I (type K or kappa) and type II (type L or lambda) polypeptides have been described (Epstein and Gross, 1964; Nachman, et al., 1965; Williams, 1964). It has been possible to delineate at least ten different antigenic determinants of the kappa type (Solomon, et al., 1965).

In addition to the antigenic differences of the types and subtypes of the light polypeptide chain, it appears that there is a unique antigenic specificity of individual light chains. This distinct specificity has been demonstrated through cross-reactions of Bence Jones antisera absorbed with pooled 7S gamma globulin (Mannik and Kunkel, 1963a). The Bence Jones proteins have been found to correspond to the light chains of both normal and myeloma immunoglobulins (Edelman and Gally, 1962; Putnam, 1962; Cohen, 1963b).

Individual light chain differences are not only demonstrated by the absence of cross reactivity with other kappa type proteins, but are, also, reflected in an analysis of the amino acid sequence of the variant portion of the polypeptide. The 212 amino acid residues (Hilschmann and Craig, 1965; Titani, et al., 1965; Milstein, 1966) of the kappa protein have been the most extensively studied. The C-terminal sequence comprising residues 106 to 212 appears to be the same for different kappa proteins with the single exception of residue 189. Variable regions of amino acid sequence occur on the N-terminal half of the chain. This portion of the chain contains residues 1 to

105. Considering all the type K Bence Jones proteins studied, 22 positions of interchange, involving 32 substitutions and 26 different pairings of amino acids have been substantiated (Titani, et al., 1966). These areas of variability have been found to be restricted to segments of the molecule around the half-cystine residue at positions 23 and 86 and the "switch peptide", constituting residues 102 and 105.

Recently, investigators have correlated certain of the polypeptide amino acid sequence variants with genetic polymorphism. Both the heavy and the light chains have been found to contain inheritable antigenic determinants reflected in structural heterogeneity. The genetic factor associated with the H-chain of the 7S immunoglobulin is designated as the Gm group, whereas the L-chains contain the Inv antigens (Kunkel, et al., 1964b; Lawler and Cohen, 1965).

The Gm factors represent a group of 14 serologically distinct groups, represented by the numbers 1 through 14 (W.H.O., 1965). The basis for the difference between a Gm (1) positive and a Gm (1) negative serum has been reported to be due to one amino acid (Baglioni, et al., 1966), while the serologic distinction of Gm (1) from Gm (4) and Gm (5) reflects a substitution of two amino acids. The Gm subunits appear to bear a direct relationship to the Ne, We, Vi and Ge H-chain subclasses (see Figure 5).

Similarly, peptide mapping has been done with the two different types of light chains, using the Bence Jones proteins. Any monocolonal gammopathy is confined to the production of one given type of light

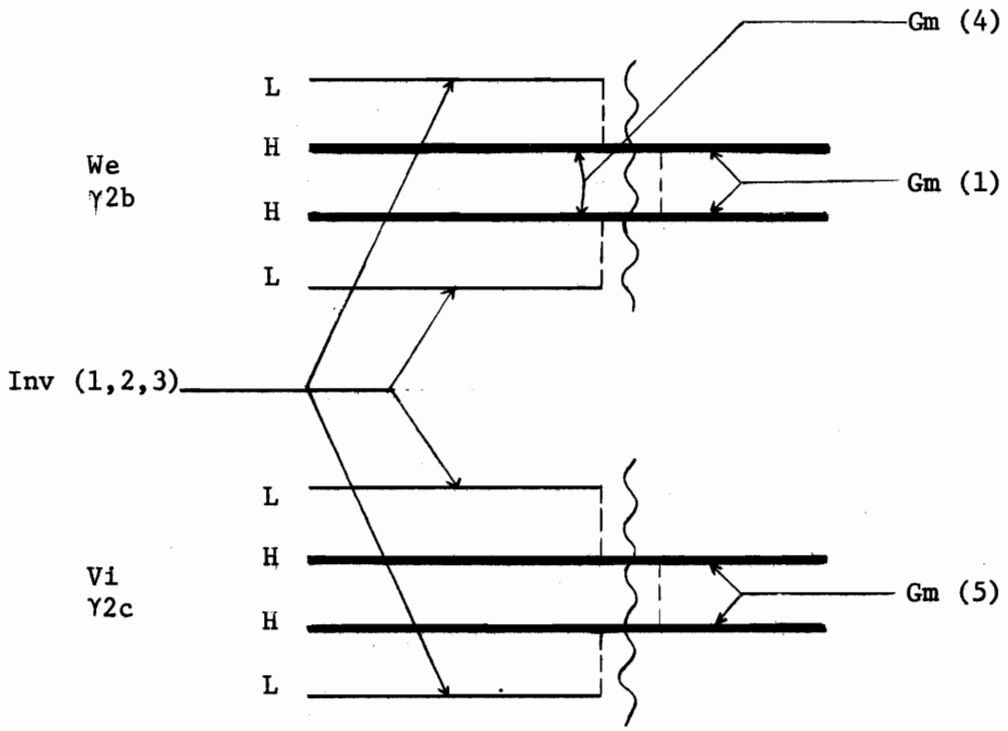


Figure 5

Schematic diagram of the chains of gamma globulin for the major We subgroup and the Vi subgroup with the approximate position of Gm and Inv factors on these chains (Kunkel, et al., 1966).

polypeptide, either of type K or type L (Bernier and Cebra, 1964; Potter, et al., 1965). Thus, it is understandable that isolated Bence Jones proteins might yield valuable information pertaining to amino acid sequence. In the analyses reported (Putnam, and Easely, 1965) there has been very little coincidence found in the tryptic peptide maps of the kappa and lambda proteins, although, an analysis of amino acid sequence has shown considerable homology in primary structure (Titani et al., 1967). This group of investigators has recently demonstrated many areas of identity of amino acid pairs when the sequence of the two types of chains are aligned. Other regions of the polypeptide chains are noted that probably differ only by the substitution of an amino acid, possibly due to single nucleotide changes within the codon.

Only one difference of current significance is seen in the terminal region at the carboxy-end of the kappa molecule. This difference involves the substitution of a single amino acid at position 189 and is directly related to the genetic determinant of the Inv group. The peptide substitution is a valine-leucine interchange. The presence of leucine is concurrent with the serologic typing of the Inv factor (2), while serologically negative Inv (2) corresponds with the presence of valine in position 189 (Baglioni, et al., 1966).

V IMMUNOGLOBULIN BIOSYNTHESIS

Basic to an understanding of the structure and function of

antibodies is the development of a concept of their biosynthesis. For many years attempts have been made to learn the mechanism involved in the selective stimulation of large quantities of a complementary antibody to a particular antigen. These studies have been subject to considerable difficulties and inconsistencies. Consequently, to date, no irrefutable model has been proposed that would coordinate the relevant findings of the investigators in this field. A number of hypothesis of antibody formation are currently a matter of conjecture (Singer and Doolittle, 1966). However, no attempt will be made here to review current concepts of the physiologic site or the biochemical process of antibody formation. Evidence concerning the cells involved in antibody production and the formation of H- and L-chains is related to the subject matter under consideration.

Immunofluorescent localization studies (Burtin and Buffe, 1963; Solomon, et al., 1963a) and radioisotopic techniques (Meyer, 1962) have demonstrated that the production of a gamma globulin component is a function of plasmacytoid and lymphoid systems. While there is ample evidence to establish the immunological competence of lymphocytes and plasmacytes, the immune mechanism is, also, characterized by the conspicuous presence of other cellular types with undefined function. These cells are the primitive reticular cells, macrophages and eosinophils (Fitch and Wissler, 1965).

Conflicting opinions exist relative to splenic involvement and antibody differentiation. The fluorescent antibody studies of

Bernier and Cebra (1964 and 1965) with human lymphoid and splenic tissue indicate that within a given cell there is a concurrent production of H- and L-chains. This synthesis was found to be limited to one of the heavy chain subclasses (γ, α, μ or δ) and to one of the light chain subgroups (κ or λ). The site of this differentiation has been confined through the utilization of radioisotopic labelling to the splenic red pulp by other investigators (Pernis and Chiappino, 1964). These data are in agreement with the earlier work of Fagraeus (1948) on splenic histology and the development of humoral antibody. The studies of Pernis and Chiappino did not show preferential synthesis in the germinal centers of the lymphoid follicles, in the white pulp of the spleen, or in the lymph nodes. Contradictory to these studies is the work with immunofluorescent staining of Mellors and Korngold (1963) and Carbonara, et al., (1963). These investigators reported the demonstration of Gamma-A in the splenic white pulp, in plasma cells of lymph nodes, and in other lymphoid tissue. The coincidental staining for both IgG and IgM was, also, reported in individual cells of the germinal centers of lymph nodes. However, Pernis and Chiappino (1964) offer a feasible explanation for this apparent contradiction in the role of splenic white pulp and splenic red pulp. They suggest that the germinal centers of the lymphoid follicles in the white pulp of the spleen and in lymph nodes may not be committed to the synthesis of a particular type of gamma globulin, whereas the individual cells of the splenic red pulp produce only one type of immunoglobulin molecule.

Mannik and Kunkel (1965) interpret the staining of Gamma-A in the lymph nodes, coupled with the in vitro radioactive incorporation of B_2A-globulins in human lymph nodes (Asofsky and Thorbecke, 1961) as evidence for the synthesis of this immunoglobulin within the lymph node. Nonetheless, immunofluorescence and radioisotopic labelling as used in all of the above studies are subject to interpretative analysis. First, allowance must be made for the presence of the immunoglobulins in interstitial fluid and for cellular interdigitation. Second, the validity of using these techniques as an index for synthesis must be taken into account. In these studies the demonstration of an immunoglobulin within a cell is assumed to denote cellular biosynthesis.

While single cells may engage in a simultaneous production of both heavy and light polypeptide components of the antibody molecule, there is evidence that quantitative differences in the formation of the two types of chains and their subgroups exist within a given cell and from cell to cell. Disproportionate heavy and light chain production becomes apparent within a single cell, i.e., the de novo production of L-chains appears to exceed the occurrence of H-chains within the same cell (Shapiro, et al., 1966). It is thought (Mannik and Kunkel, 1965) that immunologically competent cells may have the potential of producing all immunoglobulin molecules, but once differentiation synthesis occurs all other types of immunoglobulin molecules are repressed. Since individual cells preferentially synthesize only one of the polypeptides from the heavy chain subclass and one from the light polypeptide subgroup, the relative frequency

of these polypeptide types is determined by a cellular differential. Type I (kappa) cells are detected more frequently than type II (Lambda) cells (Bernier and Cebra, 1965). Of the heavy chain molecular classes, only IgA and IgG were quantitated. Gamma (IgG) containing cells appeared with a slightly greater frequency than cells containing the alpha(IgA) polypeptide.

Expected type frequencies of immunoglobulin molecules within the cellular population may be predicted from the known serum concentrations. The quantitation of the light polypeptides roughly parallels the quantitation of the serum immunoglobulin levels of kappa and lambda chains; approximately 60% of cells stained for the kappa molecule and 40% for the lambda. The differences observed in the frequency of the H-chain subclasses could not be accounted for on the basis of serum concentrations, in that the production of alpha chains was only slightly exceeded by cells containing gamma chains. Catabolic rates must, also, be given consideration, as well as the relation of the serum concentration to the total body pool (See Table 3). When the half life of these molecules is correlated with the synthetic rate, the differences in cellular occurence can be readily explained (Bernier and Cebra, 1965; Fahey and Robinson, 1963).

The hypotheses proposed to describe the nature of antigenic stimulation of antibody may be relegated into two broad groups, elective and instructive (Lederberg, 1959), on the basis of the role assigned to the antigen. The elective hypotheses depend upon the idea that antigen preferentially stimulates specific complementary

TABLE 3

SYNTHETIC CHARACTERISTICS OF THE HUMAN IMMUNOGLOBULINS

Heavy Chain Designation	Synthetic Rate (in the body)	Half Life (in the body)	Per Cent of Total Plasma Immunoglobulins	Distribution	
				Plasma	Interstitial Fluids
IgG	28 mg/kg/day	23-35 days	75-85	50%	50%
IgA	8-10 mg/kg/day	6-8 days	8-15	50%	50%
IgM	5-8 mg/kg/day	9-11 days	5-15	50-80%	30-50%
IgD	0.03-1.5 mg/kg/day	3-6 days	<.05	65-85%	15-35%
IgE	Undetermined	Undetermined	<.05	Undetermined	

Data compiled from Gitlin, 1966, and Rogentine, et al., 1966.

antibody from the gamut of inherent globulins. The instructive hypotheses consider the role of the antigen as either a direct or indirect template, acting at the antibody-synthesizing sites. Throughout the development of antibody synthesis concepts, one or the other of these hypotheses has gained favor, yet neither independently satisfactorily explains the antibody response.

Currently, instructive hypotheses are in least repute. In spite of attempts to prove or disprove the template function of antigen, the problem remains unresolved. This problem has been approached through a study of antigen binding at the protein-synthesizing sites. Wellensiek and Coons (1964), using ferritin as an antigenic stimulus, were unable to demonstrate a cellular localization of antigen in the primary response, but the secondary response was characterized by the presence of ferritin within the plasma cells. In contrast to this work are the reports of Manner et al., (1965) and Nossal et al., (1965). A study of the late stages of the 7S antibody response to I^{125}-labeled Salmonella flagella showed no antigen in the antibody-forming cell (Nossal et al., 1965). Manner's experiments were concerned with the binding of radioactive antigen to the presumed gamma-globulin-synthesizing ribosomal aggregates. Again, no significant binding was demonstrable. These experiments were unable to exclude the instructive theory, due to the possibility that antibody induction may not be invoked by the intact antigen molecule serving as a hapten and combining with a protein component to effect antibody production. Garvey and Campbell (1957) have contributed to

the hapten hypothesis by demonstrating antigen degradation in the liver, followed by the complexing of these fragments with soluble-ribonucleic acid (s-RNA). Dissociation of the fragmented antigen-s-RNA complex resulted in the loss of immunogenicity of both the antigen fragment and the s-RNA. More recently evidence has accumulated which suggests that macrophages process ingested antigen and then transfer new information concerning gamma globulin synthesis to the antibody forming cells in the form of messenger-ribonucleic acid (m-RNA) or as an RNA-antigen complex (Fishman, 1961; Fishman and Adler, 1963; Mollo et al., 1963; Nossal et al., 1964; Askonas and Rhodes, 1965). Even though there is equivocation concerning the nature of antigenic induction, there is still objection to this hypothesis, i.e., the failure to explain anamnesis (Talmage and Cohen, 1965).

Elective theories came into prominence with Burnet's (1957, 1959) modification of Jerne's (1955) hypothesis of antibody production. This introduced a genetically determined cellular basis for the elective mechanism. This postulate readily accounts for the factor unexplained by template theories, that of cellular memory; nevertheless, natural selection is not without its difficulties. Of primary concern has been the plausibility of DNA hypermutability taking precedence over accurate replication in the evolutionary origin of polypeptide antibody (Hill, et al., 1966; Singer and Doolittle, 1966; Titani, et al., 1967). The premises upon which natural selection are based have been examined, and the theory enlarged and altered in an effort to explain these inconsistencies.

Amino acid analyses have demonstrated a sharp segregation of a constant and variable portion within each of the constituent polypeptide chains of the antibody molecule. Consequently, Franklin (Fraugione and Franklin, 1965b) was prompted to propose an eight chain gamma globulin molecule to account for this synthesis. The proposal, that each of the polypeptide chains might, in fact, be two separate chains synthesized by different genes could be supported if a chemical bond linking these chains could be demonstrated. However, attempts to detect disulfide, ester or other nonpeptide bonds (Bennett, et al., 1965; Dreyer and Bennett, 1965) give no indication that subchain synthesis is involved. Nonetheless, subchain or subunit synthesis remains an attractive hypothesis to explain the amino acid sequence variability on the NH_2-terminal half of the heavy and light polypeptides. It has been further suggested that aberrance may be produced by the mode of combination of these units (Stenzel, et al., 1964). The feasibility of subunit synthesis received impetus from the demonstration of short subunit gamma globulin synthesis on relatively small ribosomal aggregates and on single ribosomes (Manner et al., 1965). However, in contradistinction to this report are the experimental results of Tawde, et al., (1966). Originally, Scharff and Uhr (1965) reported nascent protein bound to single ribosomes and small aggregates, but further investigation showed that the polyribosomes had been degraded by endogenous ribonuclease within the system. The latter report of these authors has indicated that gamma globulin synthesis takes place on polyribosomes and is dependent upon

the continued synthesis of RNA from a DNA template.

In order to construct a theory that would obviate the improbability of hypermutability, certain facets of the molecular basis of antibody formation must be recognized. A few of these considerations are enumerated: 1) The consistency in replication of the amino acid sequence of the carboxy-terminal half of the heavy and light polypeptides would indicate that this portion of each of the chains was either the product of a single gene, or the product of two or more genes derived by gene duplication of a common ancestor. Whereas, the gross heterogeneity at the amino-end of these polypeptides could be produced from innumerable other genes. 2) The immunoglobulin antigenic determinants do not appear to be inherited as alleles. This is because, for the most part, each antigen is present in all individuals and is expressed in the serum at a constant level (Mannik and Kunkel, 1963b). 3) The antigenic determinants are probably characterized by separate genetic loci and are not subject to segregation, as evidenced by their presence in a consistent ratio. For example, kappa and lambda chains are found in a 3:2 ratio in plasma cells and in the serum. 4) It has been indicated that the structural cistrons for certain of the antibody heavy chain phenotypes are adjacent, or are in close proximity on the same chromosome (Herzenberg, 1964; Lieberman, et al., 1965). 5) In relation to gene information transfer, any functional theory of antibody synthesis must provide a mechanism for the DNA or RNA code to be formulated from the antigenic stimulus and a mechanism for subsequent regulation.

Other hypotheses have been advanced that would replace or account for the apparent phenomenon of hypermutability. Smithies (1963) has proposed a multiple crossing over scheme between the strands of nucleic acids that code for the variable portions of the molecule. Another suggestion (Potter, et al., 1965) is that the different amino acid sequences present at the active site region could be regulated by unusual triplets located at key positions on particular portions of the molecule and could be read alternately by different acceptor RNA molecules with independent specificities. Dreyer and Bennett (1965) would explain peptide variances by an insertion of an aberrant section into the uniform region of the amino acid sequence by a mechanism analogous to the association of the λ-virus with the Escherichia coli chromosome.

Perhaps of most significance in elucidating the enigma of seeming erratic polypeptide replication are the recent studies of Hill, et al., (1966), Singer and Doolittle (1966), and Titani et al., (1967). These authors reported a chemical homology between the Fc-fragment of the heavy chain and the invariant half of the kappa light chain. A chemical relationship between the variant half of the light chain and the Fd-piece from the heavy chain was also suggested. Furthermore, these studies indicate hypermutation is probably not the functional mechanism involved in antibody heterogeneity, but that heterogeneity may have resulted from the accumulation of one step mutations in multiple genes which were originally derived by an evolutionary process involving gene duplication from a single primitive gene.

VI THE MYELOMATOSES

Classically, multiple myeloma is defined as a neoplastic proliferation of plasma cells with an elaboration of an M component in the serum and/or urine, and multiple skeletal lytic lesions, frequently in association with anemia, hypercalcemia, coagulation disorders and renal impairment (Osserman, 1965). The designation, M component, was introduced by Riva (1957) to denote structurally unique, homogeneous proteins in pathologic states that are increased in the serum or urine. Structurally, these abnormal proteins resemble the "normal" immunoglobulins to such an extent that it is difficult to state with any certainty whether the M component represents a hyperplastic synthesis of a finite globulin from the gamut of inherent globulins, or consists of an aberrant synthesis of an abnormal protein (Edelman and Poulik, 1961; Edelman et al., 1961). In any event, it is evident that the capacity to produce functional antibodies is impaired (Lawson, et al., 1955). Usually, the presence of an M component from one of the heavy chain subclasses is associated with a pronounced decrease of all other immunoglobulin classes (Osserman, and Lawlor, 1961). The mechanism for the synthesis of the immunoglobulins in the myelomatoses may be considered to be disrupted both from the standpoint of the production of a functional antibody, and in the maintenance of an equilibrium between the catabolic and anabolic rates of these globulins. The hypothesis may be formed that some process exists which preferentially favors synthesis in the

neoplastic cell line and which depresses the activity of all other existing gamma globulin-synthesizing cells.

The myelomatoses have been classified according to the polypeptide compositions of the M component (see Table 4). Abortive synthesis of the antibody molecule may occur as an excessive cellular proliferation of the light polypeptide constituent, as a disproportionate increase in heavy chain production, or the neoplastic cell line may produce a whole myeloma protein containing both heavy and light chains. When the M component consists of heavy chains in combination with light chains, the myeloma is classified in accordance with the antigenic typing of the heavy chain determinant. The most recently described myelomatosis of this classification is Gamma-D (Rowe and Fahey, 1965a). Gamme-E protein has been described (Ishizaka, et al., 1966), but has not, as yet, been implicated in the myelomatoses. The term "light chain disease" has been used to describe the presence of free L-chains in the serum (Williams, et al., 1966). This disease was first recognized as a hypogammaglobulinemia with a continual absence of serum M components by paper or cellulose acetate strip electrophoresis. Elevated serum levels of free L-chains may be detected by immunodiffusion; however, this finding is not confined to hypogammaglobulinemic states of myeloma. A third state where aberrant proteins are produced is known as "heavy chain disease" (Franklin, et al., 1964). The first observed instance of an excess production of the IgG heavy polypeptide chain was reported in 1964. The presence of large quantities of the IgA and IgM heavy polypeptide determinants

TABLE 4

ABNORMAL IMMUNOGLOBULIN SYNTHESIS

CLASSIFICATION	MOLECULAR STRUCTURE	MONOCLONAL SERUM	PEAK URINE	BENCE JONES PROTEIN	FREE SERUM L-CHAINS
1. Multiple Myeloma		X	X	X	±
γA Type	$\alpha\ \lambda\ \alpha\ \kappa$				
γG Type	$\gamma^2\ \lambda^2\ \gamma^2\ \kappa^2$				
γD Type	$\delta^2_2\ \lambda^2_2\ \delta^2_2\ \kappa^2_2$				
	λ or λ_2 L-chains				
	κ or κ^2_2 L-chains				
(As above without L-chains)		X	-	-	-
Bence Jones Type (Normoproteinemic)		-	X	X	±
	λ or λ_2 L-chains				
	κ or κ^2_2 L-chains				
2. Macroglobulinemia	$(\gamma M)\ (\mu_2 \lambda_2)n;\ (\mu_2 \kappa_2)n$	X	-	-	±
3. H-Chain Disease	γ - H-Chains	X	X	-	-
4. L-Chain Disease	λ or λ_2 L-chains	-	X	X	X
	κ or κ_2 L-chains				

Adapted from Cawley, 1966.

in the serum or urine is yet to be described. A relative distribution of the immunologic types of gammopathies is presented in Table 5.

As may be seen from Table 5, the presence of Bence Jones protein is frequently a characterizing parameter of the myelomatoses. Possibly, because of the small molecular size of the light chains, these proteins are cleared in the kidney and appear in the urine without apparent degradation. This allows for the categorizing of these proteins into one of the two serologically distinct light chain subgroups, kappa or lambda. As a general rule, patients who exhibit kappa type myeloma proteins in the serum also excrete the kappa Bence Jones protein. Likewise, lambda type Bence Jones proteins are usually associated with lambda myeloma proteins (Mannik and Kunkel, 1962; Migita and Putnam, 1963).

TABLE 5

APPROXIMATE DISTRIBUTION OF IMMUNOLOGIC TYPES

OF PLASMACYTIC DYSCRASIAS

Classification	Per Cent Of Total	Per Cent With Bence Jones
1. Multiple Myeloma		
Hypergammaglobulinemic		
Gamma-A Type	20-25	30
Gamma-G Type	50-55	30
Normoproteinemic (Bence Jones)		
Gamma-D Type	3	100
L-Chain Disease	5-20	100
Unclassified	2	100
2. Macroglobulinemia	10-20	10
3. H-Chain Disease (five reported cases)		0
4. Unclassified	1.5	

MATERIALS AND METHODS

I SERA

Fresh and stored serum samples were studied from patients with the clinical diagnosis of multiple myeloma at the Latter Day Saints Hospital. Blood samples were collected and allowed to clot at 37°C. The samples were centrifuged and the serum was separated. The sera were preserved by adding either powdered merthiolate (Thimerosal, NF, Eli Lilly & Co.) or sodium azide to give a final concentration of 0.1% (w/v). The specimens were then refrigerated at 4°C or frozen at -20°C until serologic procedures were performed.

II URINE

Twenty-four-hour urine samples from patients with multiple myeloma were collected in refrigerated bottles containing merthiolate as a preservative. The urine specimens were clarified by centrifugation and a portion of the twenty-four-hour sample was concentrated 10 to 100 fold by dialysis against a 30% (w/v) polyvinyl-pyrrolidone (PVP, General Aniline & Film Corp.) solution made isotonic with sodium chloride. In some instances, another portion of urine was mixed with a saturated solution of ammonium sulfate and the precipitate was dialyzed against distilled water and finally against 30% PVP.

III CELLULOSE ACETATE ELECTROPHORESIS

Electrophoretic separation and quantitation of serum and urine

protein fractions were performed on cellulose acetate strips using a micro system (Beckman Instruments, Fullerton, California).

A. Veronal Buffer

A veronal buffer solution, pH 8.6, ionic strength 0.075, was prepared according to the following formula: 2.76 g 5,5-diethyl-barbituric acid and 15.40 g sodium 5,5-diethylbarbituric acid in distilled water to make 1 liter.

B. Protein Stains

Two dye solutions were prepared. One, a routine protein stain (Ponceau-S), was used as a primary stain on cellulose acetate membranes containing both urine and serum protein samples. The second dye (Nigrosin) was used as a counterstain because of its greater affinity for proteins and was required only in staining for low protein concentrations in the urine preparations. Ponceau-S dye fixative solution contained 0.2% (w/v) Ponceau-S stain, 3.0% (w/v) trichloroacetic acid and 3.0% (w/v) sulfosalicylic acid in water. The Nigrosin stain was made to 0.002% (w/v) in aqueous acetic acid.

C. Procedure

Cellulose acetate membranes saturated with buffer were mounted on the Microzone ® electrophoretic cell bridge and serum samples (.0003 ml) or urine samples (.0003 to 0009 ml) were applied. Electrophoresis was performed at a constant current of 5 milliamperes (ma) for twenty minutes at room temperature. The membrane was removed from the bridge and immersed in a Ponceau-S fixative dye solution for 10 minutes. The membrane was then transferred to a 5% aqueous acetic

acid rinse until the background was clear. The membranes containing the serum samples were placed in an absolute methanol rinse for 1.5 minutes and transferred to a clearing solution of 20% glacial acetic acid in absolute methanol for 1.5 minutes. While still in solution, the membranes were positioned over glass plates. The membranes attached to the glass plates were dried at 100°C for ten minutes. The transparent membranes were then inserted into protective plastic envelopes, scanned and quantitated on the Analytrol® equipped with microzone attachment (Beckman Instruments, Fullerton, California). A B-a optical density cam, a 500 mu interference filter in the rear position, and a 520 mu filter in the front position were used on the Analytrol with the slit width selector completely open.

The procedure in staining and quantitating the urinary proteins was the same as for the serum proteins, except for the following additional steps: After the last 5% aqueous acetic acid rinse the membranes were transferred to a Nigrosin stain and left in this solution overnight. The stained membranes were then again taken through the acetic acid rinses.

IV IMMUNOELECTROPHORESIS

A. Antisera

All antisera used throughout these studies were procured from Hyland Laboratories, Los Angeles, California. The polyvalent antisera were commercial preparations obtained from animals (horse, goat, and rabbit) hyperimmunized with pooled normal human sera. Commercial

immunoglobulin class specific (IgG, IgA and IgM) antisera and light chain (kappa and lambda) antisera were prepared by animal hyper-immunization with an appropriate paraprotein.

B. Veronal Buffer

1. Tank Buffer

The tank buffer solution was made according to the following formula: 1.38 g 5,5-diethylbarbituric acid, 8.7 g sodium, 5,5-diethylbarbiturate and 0.39 g calcium lactate in 1 liter of distilled water.

2. Agar Buffer

The agar buffer was made according to the following formula: 1.66 g of 5,5-diethylbarbituric acid, 10.51 g sodium 5,5-diethyl-barbiturate and 1.54 g calcium lactate in 1 liter of distilled water.

C. Preparation of Agar Buffer

Agar was obtained from two sources (Difco Laboratories and Bausch & Lomb). Agarose (Bausch & Lomb, Rochester, New York) is a low-ion agar that has the agaropectin moiety of agar removed and needed no further purification. Bacto Noble Agar (Difco Laboratories, Detroit, Michigan) was purified by the following process: The agar was allowed to swell overnight in a large volume of distilled water, followed by a thorough washing in distilled water by means of repeated decantation of the supernatant from the settled agar and replacement of distilled water until a clear supernatant was obtained. When the supernatant was colorless the distilled water was decanted and the agar dehydrated with alcohol. The agar was then rinsed with acetone, taken to dryness

on a Buchner funnel and allowed to come to complete dryness at room temperature.

To prepare the buffered agar medium, purified agar was made 2% (w/v) in water and the solution slowly brought to a boil. Bacterial growth was inhibited by the addition of sodium azide to a final concentration of 0.1% (w/v). The agar solution was allowed to solidify in 50 ml aliquots. On the day of use, this material was remelted in a boiling water bath and then cooled to 56°C. A 50 ml aliquot of diethylbarbituric buffer heated to 56°C was added to the hot agar solution and thoroughly mixed.

D. Procedure

The microtechnique of immunoelectrophoresis (Scheidegger, 1955) as modified by Hirschfeld (1960) carried out on LKB Immunophor® equipment (LKB Instruments, Inc., Washington, D.C.) was used for characterization of the serum proteins. Twenty ml of melted agar-buffer was applied to a horizontal frame containing six microscope slides previously cleaned with alcohol and coated with agar by immersion in a boiling solution of 0.2% (w/v) purified agar. The agar-buffer was allowed to solidify. Just prior to use, the slides were punched with a die which cut antigen wells and antibody trenches into the agar.

The slide frames were mounted on the electrophoresis cell and connected to the buffer in the electrode vessels with rayon wicks soaked in the tank buffer.. The lid was placed on the electrophoresis cell and 35 ma constant current was applied for 15 minutes. The cell

was opened and the agar from the antigen wells was aspirated. Five to ten microliters (µl) of serum or urine was pipetted into the antigen wells. The same current was again applied for approximately one hour, or until the albumin fraction of normal human serum, labelled with bromphenol blue, migrated 2.5 cm.

At the discontinuance of the electrophoretic separation, the agar was removed from the antibody trenches and 0.1 ml of antisera was added. After application of the antisera, the slides were placed in a moist diffusion chamber and incubated at room temperature for approximately 24 hours. The slides were cleared of nonprecipitated protein and buffer salts by washing in three 24 hour rinses of 1% saline, followed by a 24 hour rinse in distilled water. The slides were dried in a hot-air dryer and then placed in the staining solution for 15 minutes. Excess stain was removed by several brief rinses in cold tap water. The final differentiation was carried out in 5% aqueous acetic acid. The stained slides were dried, labelled and photographed.

V IMMUNOGLOBULIN QUANTITATION

The immunoglobulins (IgG, IgA and IgM) were quantitated by radial immunodiffusion, using buffered agar gel plates impregnated with a specific immunoglobulin antisera (Hyland Laboratories, Los Angeles, California). The immunodiffusion was carried out in rectangular disposable polystyrene plates with snap-on lids. These plates contained 4 ml of agar and had six wells of equal size (1-2 mm. in

diameter) cut equidistant in a horizontal pattern across the agar. Twenty lambda of antigen (reference standard, urinary concentrate or serum) was pipetted into each of the wells. The IgA and IgM antibody containing plates were then incubated in a moist chamber at room temperature for 16 hours. The IgG plate was incubated for 4 hours at 37°C.

At the termination of the immunodiffusion, the radius of the precipitation around the well was measured under a calibrated magnifying glass. A standard curve was constructed for each of the immunoglobulin classes from the radii of the reference samples. The radius of the immunodiffusion has been found to be directly proportional to the concentration of the antigen. The unknown samples were quantitated by interpolation of the calibration curve.

VI PROTEIN QUANTITATION

An estimation of the serum and urinary proteins was made colorimetrically by the biuret method (Campbell et al., 1963). The biuret reagent was made by dissolving each of the following reagents in about 500 ml. distilled water: 3.0 g $CuSO_4 \cdot 5H_2O$, 12.0 g $KNaC_4H_4O_6 \cdot 4H_2O$ and 60.0 g NaOH. The sodium potassium tartrate solution was added to the sodium hydroxide solution and mixed. The cupric sulfate solution was then slowly added to this mixture and the final volume was brought to 2 liters.

Analysis was made according to the following procedure: 0.2 ml of sample, blank and standard was added to 2.8 ml of 0.85% NaCl and

mixed on the Vortex Mixer® (Clay-Adams, Inc., New York). Biuret reagent (3.0 ml) was then added to each tube and immediately mixed. Protein standards, calibrated by Kjeldahl analysis, were obtained from Armour Co., Chicago and from Hyland Laboratories, Los Angeles, California. All tubes were incubated at room temperature for 30 minutes and read at a wave length of 520 millimicrons (mμ) on a spectrophotometer. The protein content was calculated from a standard curve.

VII DETECTION OF BENCE JONES PROTEINURIA

A. Non-Specific Proteinuria Screening Tests

Two routine protein screening methods were used to detect the presence of albumin, globulin, Bence Jones protein or proteoses.

1. Paper Strip Indicator Test

Fresh urine samples were tested with a citrate buffered (pH 3.5) tetrabromphenolsulfonphthalein impregnated filter paper strip (Combistix, Ames Co., Elkhart, Indiana). An indication of protein content was obtained by comparison with the color scale provided with the indicator strips.

2. Sulfosalicylic Acid Test (magnus-Levy, 1936)

Protein was detected by the following procedure: Four drops of 20% (w/v) sulfosalicylic acid was added to approximately 2 ml of centrifuged urine and mixed. If no precipitation occurred, an excess of sulfosalicylic acid was then added to ensure protein detection in alkaline urines. Proteinuria was indicated by the presence of a

precipitate in the acidified urine.

B. Specific Tests For Bence Jones Proteinuria

Bence Jones protein testing was accomplished by an acid precipitation with p-toluene sulfonic acid (Cohen and Raducha, 1962) heat and acetic acid test (Sankuhler, 1952; Putnam, et al., 1959) and a protein-paper-extraction test (Naumann, 1965).

1. p-Toluene Sulfonic Acid (TSA) Test

The following procedure was used: 1.0 ml. of 12% TSA (w/v) in glacial acetic acid was mixed with 2.0 ml. of centrifuged urine. The presence of Bence Jones protein was indicated by the formation of a precipitate after 5 minutes.

2. Heat And Acetic Acid Test

The following technique was used: About 10.0 ml of centrifuged urine was adjusted to a pH of 4.9 with a dilute acetic acid solution and heated in a water bath. The temperature reading was observed on a thermometer in the tube of urine. The urine was brought to a boiling point and boiled for at least 5 minutes. While still near the boiling point, the urine was filtered and the filtrate was observed for precipitation upon cooling. Clouding of the filtrate at 85 to 65^{o}C was read as a positive test.

3. Protein-Paper-Extraction Test

This procedure was developed to differentiate uroglobulins and proteoses from Bence Jones proteinuria. The test was performed as follows: The uroproteins were precipitated by adding 1.0 ml of 50% (w/v) trichloracetic acid to 10.0 ml. of centrifuged urine. After

precipitation, the solution was centrifuged and the supernatant was decanted. One to two drops of 27% ammonium hydroxide was added to the precipitate. The latter was mixed and pulverized with a glass stirring rod. Approximately 0.1 ml. of this suspension was absorbed onto a 1/8 sector of 9 cm circular filter paper (Whatman No. 1). The paper was dried and the Bence Jones protein eluted in 1.5 ml. boiling sodium chloride-acetate buffer, pH 4.3. The buffer was composed of 100 ml. 10% NaCl, 14.0 ml. 2 M sodium acetate, 36.0 ml. 2 M acetic acid and distilled water to make a final volume of 2 liters. The buffer solution was allowed to cool for 10 minutes, then the tube was immersed in cooled water, the filter paper removed from the tube and 3 drops of 20% (w/v) sulfosalicylic acid was added. The presence of Bence-Jones protein was indicated by a clouding of the buffer solution.

VIII BENCE JONES PROTEIN CHARACTERIZATION

Bence Jones proteins were classified as kappa type or lambda type by immunoelectrophoresis, using specific antisera (see Section IV).

EXPERIMENTAL RESULTS

Serum and urinary proteins of twenty-six patients under treatment for a myelomatous disease were studied at the L.D.S. Hospital in Salt Lake City, Utah. These patients were selected on the basis of serum electrophoretic patterns, thermal solubility properties of the urinary proteins, and the availability of urine and serum samples for protein studies. In all cases, the presence of a myelomatous process was confirmed by the clinician through an evaluation of the clinical and pathologic manifestations of the patient's disease.

The abnormal myeloma proteins found in this study were characterized by a considerable diversification in the quantitative elaboration of an M-component, the range in electrophoretic mobility, and the heterogeneity in the underlying molecular abnormality of gamma globulin. An arbitrary classification of the gammopathies was made in accordance with the clinical and laboratory findings listed in Table 4. Analyses for free serum L-chains were not performed, nor were macroglobulinemia, H-chain disease and L-chain disease included in this study. Of the twenty-six gammopathies studied (see Table 6), five were normoproteinemic myelomas, four Gamma-A paraproteinemias, two Gamma-D paraproteinemias, and fifteen Gamma-G paraproteinemias. In this paper, normoproteinemic myeloma has been used to refer to myelomas wherein a study of the urinary proteins was essential to establish the presence of an M-protein, since no evidence of an abnormal protein was discernible in the serum

TABLE 6

ELECTROPHORETIC FRACTIONATIONS OF SERUM PROTEINS

Myeloma Type and Patient Designation	Total Protein	Protein Concentrations (gm/100 ml) Albumin	α-1 Globulin	α-2 Globulin	B-Globulin	γ-Globulin
Normal (Mean ± 1 S.D.)	7.1 ± 0.30	5.03 ± 0.32	0.18 ± 0.08	0.46 ± 0.09	0.52 ± 0.12	0.92 ± 0.11
Normoproteinemic						
Patient A	6.3	3.23	0.30	0.71	1.05	1.01
Patient B	6.7	4.10	0.43	0.92	0.70	0.54
Patient C	6.7	4.54	0.21	0.55	0.72	0.68
Patient D	6.3	3.62	0.33	0.87	0.54	0.94
Patient E	4.9	2.50	0.39	0.74	0.57	0.70
Gamma-A						
Patient F	9.7	3.60	0.30	0.96	0.67	4.18
Patient G	7.7	2.35	0.52	1.41	2.69	0.73
Patient H	9.6	3.38	0.39	0.90	4.53	0.40
Patient I	9.4	3.30	0.42	0.35	4.85	0.40
Gamma-D						
Patient J	6.1	3.80	0.18	0.57	0.84	0.71
Patient K	5.7	2.53	0.23	0.74	0.60	1.61

TABLE 6--Continued

ELECTROPHORETIC FRACTIONATIONS OF SERUM PROTEINS

Myeloma Type and Patient Designation	Total Protein	Protein Concentrations (gm/100 ml) Albumin	α-1 Globulin	α-2 Globulin	B-Globulin	Y-Globulin
Gamma-G						
Patient L	10.8	2.89	0.35	0.81	0.89	5.85
Patient M	7.1	4.60	0.21	0.84	0.39	1.06
Patient N	12.0	3.87	0.36	0.43	0.74	6.50
Patient O	14.2	6.01	0.17	0.83	1.00	6.18
Patient P	9.2	3.08	0.13	0.68	0.78	4.53
Patient Q	8.5	3.91	0.28	0.61	0.67	3.02
Patient R	9.0	3.24	0.50	0.84	0.84	3.58
Patient S	6.3	2.93	0.46	0.77	0.66	1.48
Patient T	8.1	2.88	0.39	0.83	1.26	2.74
Patient U	10.6	2.92	0.46	0.96	1.43	4.82
Patient V	7.8	1.44	0.23	1.10	1.49	3.54
Patient W	10.6	3.26	0.42	0.86	0.97	5.01
Patient X	6.7	3.45	0.35	1.24	0.83	0.83
Patient Y	7.2	3.51	0.36	0.99	0.45	1.89
Patient Z	14.8	4.19	0.46	0.64	0.73	8.76

TABLE 7

ELECTROPHORETIC FRACTIONATIONS OF CONCENTRATED URINARY PROTEINS

Myeloma Type and Patient Designation	Total Protein (gm/100 ml)	Percent Protein Concentration				
		Albumin	α-1 Globulin	α-2 Globulin	B-Globulin	γ-Globulin
Normal (Normal levels have not been determined)						
Normoproteinemic						
Patient A	---	---	---	----	----	----
Patient B	---	6.5	0.5	4.0	89.0	+
Patient C	---	13.0	7.0	20.0	57.0	3.0
Patient D	---	17.0	7.0		8.0	68.0
Patient E	---	6.0	2.0	2.0	28.0*	62.0
Gamma-A						
Patient F	---	23.0	7.0	12.0	43.0*	15.0
Patient G	4.3	16.0		14.0		70.0
Patient H	7.5	7.0	3.0	1.5	4.5	84.0
Patient I	7.5	10.5	5.5		10.5	73.5
Gamma-D						
Patient J	---	---	---	----	----	----
Patient K	---	3.5	6.5			90.0

TABLE 7--Continued

ELECTROPHORETIC FRACTIONATIONS OF CONCENTRATED URINARY PROTEINS

Myeloma Type and Patient Designation	Total Protein (gm/100 ml)	Albumin	Percent Protein Concentration α-1 Globulin	α-2 Globulin	B-Globulin	γ-Globulin
Gamma-G						
Patient L	---	9.5	_____ 5.0 _____		0	85.5
Patient M	---	3.5	_____ 6.0 _____		0	
Patient N	---	25.0	1.0	67.0	2.5	4.5
Patient O	---	9.0	5.5	16.0	26.0	43.5
Patient P	---	16.5	7.0	38.0	29.0	9.5
Patient Q	---	34.0	____ 34.0 _____		21.0	11.0
Patient R	---	----	---	----	----	----
Patient S	---	----	---	----	----	----
Patient T	---	----	---	----	----	----
Patient U	---	----	---	----	----	----
Patient V	---	----	---	----	----	----
Patient W	9.0	27.0	23.0	10.5	5.0	24.5
Patient X	---	----	---	----	----	----
Patient Y	---	----	---	----	----	----
Patient Z	--- *	14.0	_____ 8.0 _____		66.5	11.5

* Indicates denatured protein

\+ The solid line used in the chart indicates the range of electrophoretic mobility of the given protein. A precise mobility could not be designated in as much as the peak was not sharp and could not be differentiated.

electropherogram. Final classification was based on serologic analyses by immunoelectrophoresis.

I TOTAL SERUM PROTEINS

The term "hyperproteinemia" is frequently associated with the myelomatous process. There is no concordant opinion as to the precise serum protein level to which this term is applicable. The only consensus seems to be that in hyperproteinemia, the total protein value must exceed 8.0 gm/100 ml (Jeghers and Selesnick, 1937; Gutman, et al., 1941; Wuhrman and Marki, 1963; Hart, et al., 1965). However, the myelomatous process may also be characterized by normoproteinemic and hypoproteinemic states. The Gamma-D myelomas (Hobbs, et al., 1966) are primarily associated with normoproteinemia, while the Bence Jones myelomas (Solomon and Fahey, 1964) are usually associated with hypoproteinemia, or normoproteinemia. In the series of twenty-six myelomatoses presented herein, thirteen cases were found with serum protein values of less than 8.0 gm/100 ml (see Table 6). In the normoproteinemic series one patient had a total protein value as low as 4.9 gm/100 ml. The average value for the normoproteinemic series was 6.2 gm/100 ml. Of the remaining classifications, the Gamma-A series of patients were characterized as possessing an average value of 9.1 gm/100 ml; the average value of the Gamma-D group was 5.9 gm/100 ml and the average for the Gamma-G group was 9.5 gm/100 ml. In general, the total protein values for the the individual cases deviated little from the average of each myeloma

classification, with the exception of the Gamma-G group. The range of the total proteins in this group was 6.3 gm/100 ml to 14.8 gm/100 ml. Five of the patients within the Gamma-G series had total protein values less than 8.0 gm/100 ml.

II CELLULOSE ACETATE ELECTROPHORESIS

Serum electrophoretic fractionation was used in conjunction with other laboratory tests as a means to detect paraproteins. Paraproteins have been defined as proteins in the serum, urine or tissues produced by myeloma cells (Apitz, 1940). These proteins may be demonstrated electrophoretically as dense staining bands in the globulin region. Quantitation of these abnormal proteins has been determined by densitometric analyses. The dense protein zone seen on the cellulose acetate medium has been found to correspond with the "peak" or "spike" produced on the electropherogram (Cawley, 1966). These protein patterns may appear as thin, homogeneous spikes or as additional peaks in a globulin region where under "normal" circumstances only one peak is observed. Figure 10 shows three gamma globulin peaks in an area where there is usually only one peak. The presence of a paraprotein in the serum does not constitute a diagnosis of myeloma. Approximately 10 per cent of patients with demonstrable serum paraproteins do not have an associated neoplasia (Cawley, 1966). Benign pseudo-M-proteins must be differentiated from true gammopathies by other laboratory examinations and by long-term follow-up studies. Preclinical myeloma may be indicated by the presence of a

paraproteinemia in the electrophoretic pattern as early as seventeen years in advance of the development of clinical myeloma (Norgaard, 1964). As previously stated, the electrophoretic detection of a paraprotein was confirmed as a manifestation of the myelomatous process before being included in this study.

Electrophoretic separation of the proteins of the myeloma sera and urine was performed primarily on a cellulose acetate medium. The mobility of each fraction was determined and the concentration calculated. Table 6 and Table 7 are compilations of these data. The patients are grouped according to the immunoglobulin class of the paraprotein. Representative and unusual electrophoretic patterns from each of the myeloma classes studied are given in Figure 6 through Figure 12.

In the normoproteinemic series, no abnormal globulin was evident in the sera; however, in almost every instance, a pronounced protein component was found in the urine (see Tables 6 and 7). Immunologic studies of these components are described in Section VI. Figure 6 shows the serum and urine electropherograms from a representative patient in this group.

An examination of the electrophoretic patterns of the Gamma-A type myelomas showed that the mobility of the M-protein in the serum ranged from the gamma globulin region through the beta globulin region, with a predominance in the latter. In all but one instance (see Table 6) the concentration of M-protein was above 4.0 grams per cent. The mobility of the M-proteins in the urine also ranged

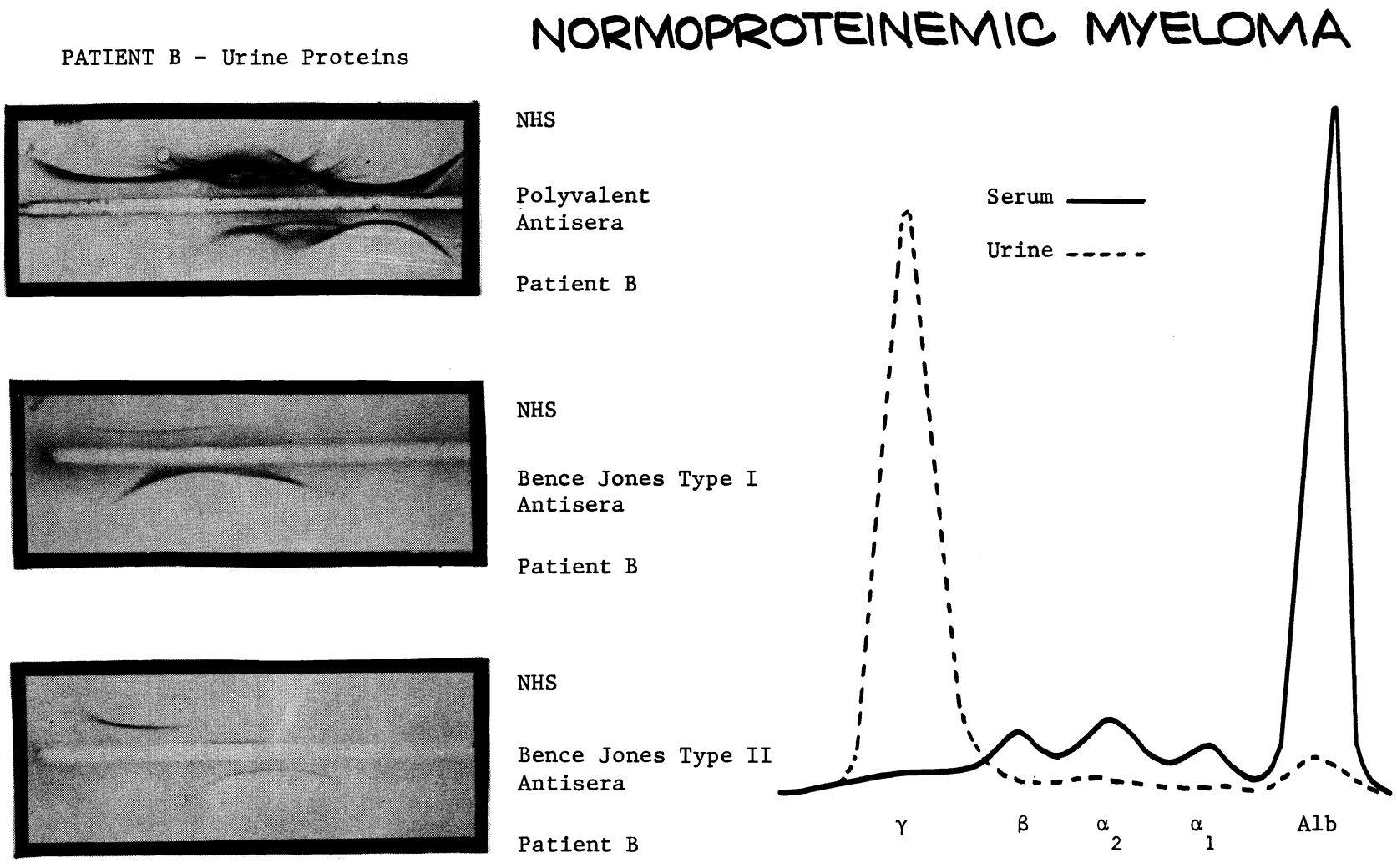


Figure 6. Electrophoretic and immunoelectrophoretic patterns of serum and urine proteins. A normal human serum pattern, designated "NHS", is shown at the top of the immunoelectrophoresis for comparison. The antisera used in the immunoelectrophoresis is indicated to the right of the photograph.

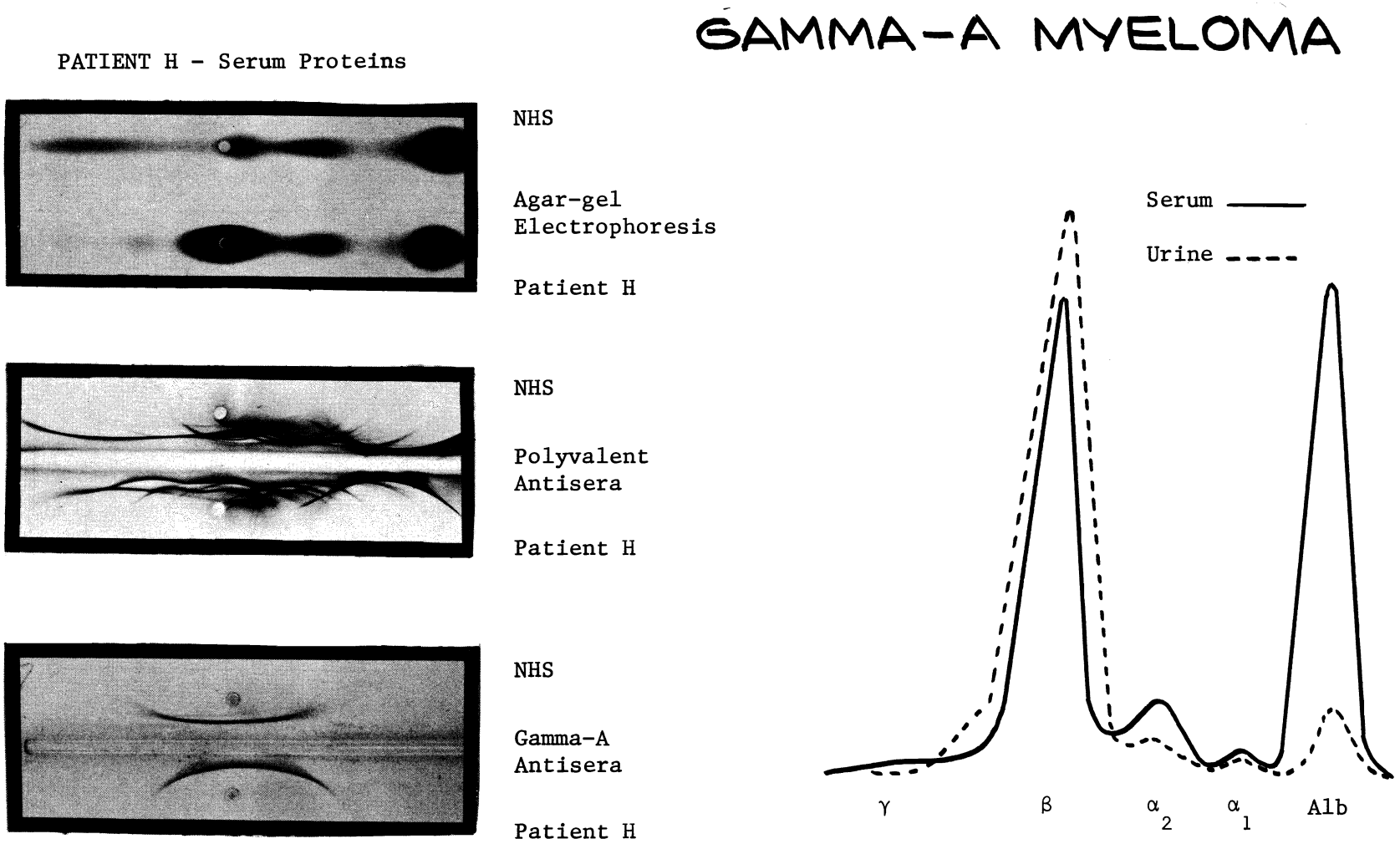


Figure 7. Electrophoretic and immunoelectrophoretic patterns of serum and urine proteins. A normal human serum pattern, designated "NHS", is shown at the top of the immunoelectrophoresis for comparison. The artisera used in the immunoelectrophoresis is indicated to the right of the photograph.

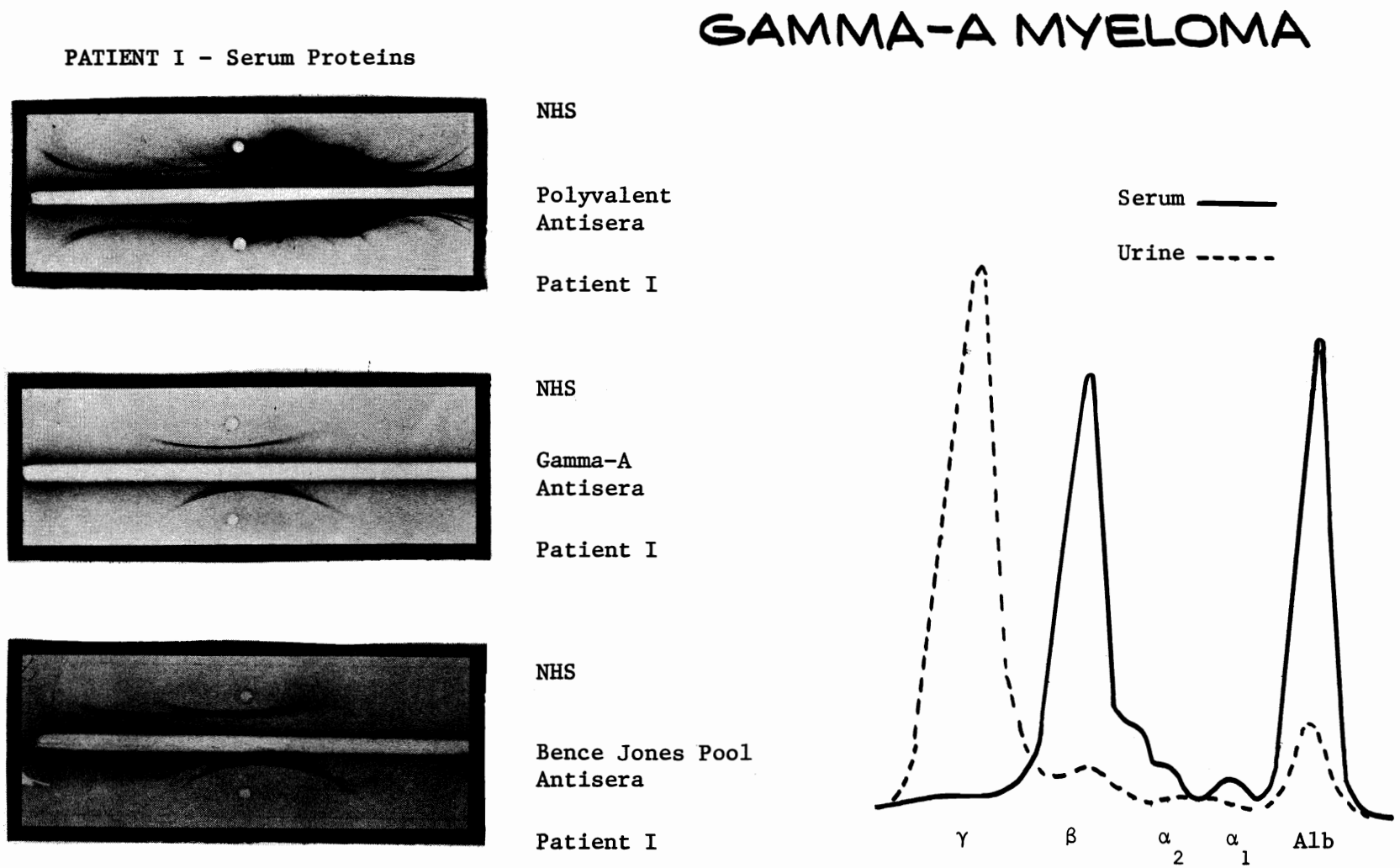
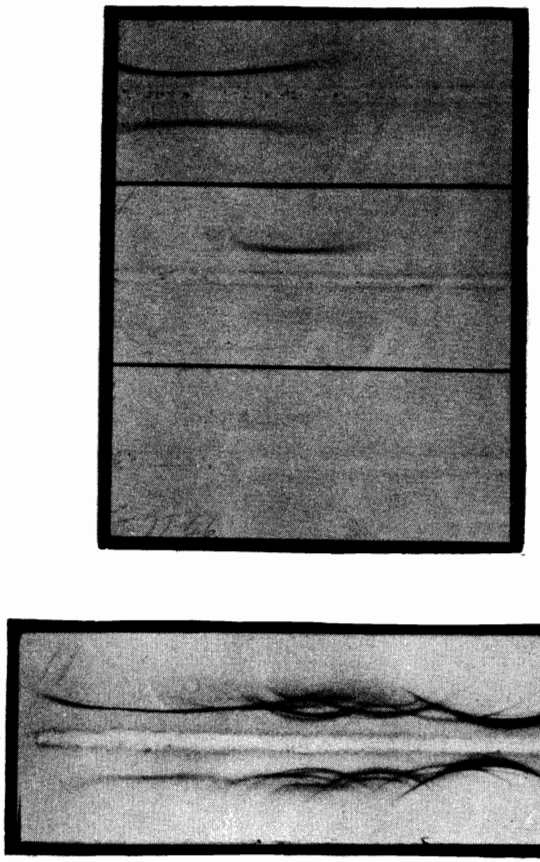


Figure 8. Electrophoretic and immunoelectrophoretic patterns of serum and urine proteins. A normal human serum pattern, designated "NHS" is shown at the top of the immunoelectrophoresis for comparison. The antisera used in the immunoelectrophoresis is indicated to the right of the photograph.

PATIENT K - Serum Proteins



NHS

Gamma-G Antisera

Patient K

NHS

Gamma-A Antisera

Patient K

NHS

Gamma-M Antisera

Patient K

NHS

Polyvalent Antisera

Patient K

GAMMA-D MYELOMA

Serum ——

Urine - - - - -

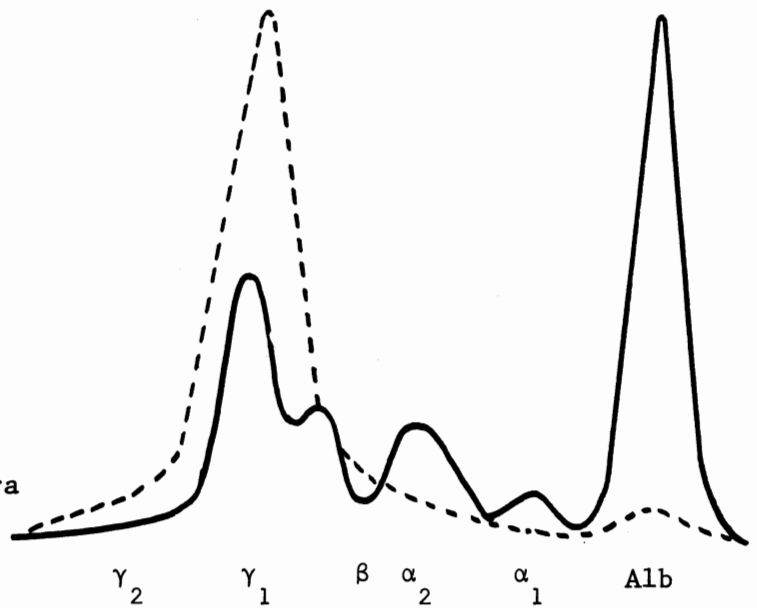


Figure 9. Electrophoretic and immunoelectrophoretic patterns of serum and urine proteins. A normal human serum pattern, designated "NHS", is shown at the top of the immunoelectrophoresis for comparison. The antisera used in the immunoelectrophoresis is indicated to the right of the photograph.

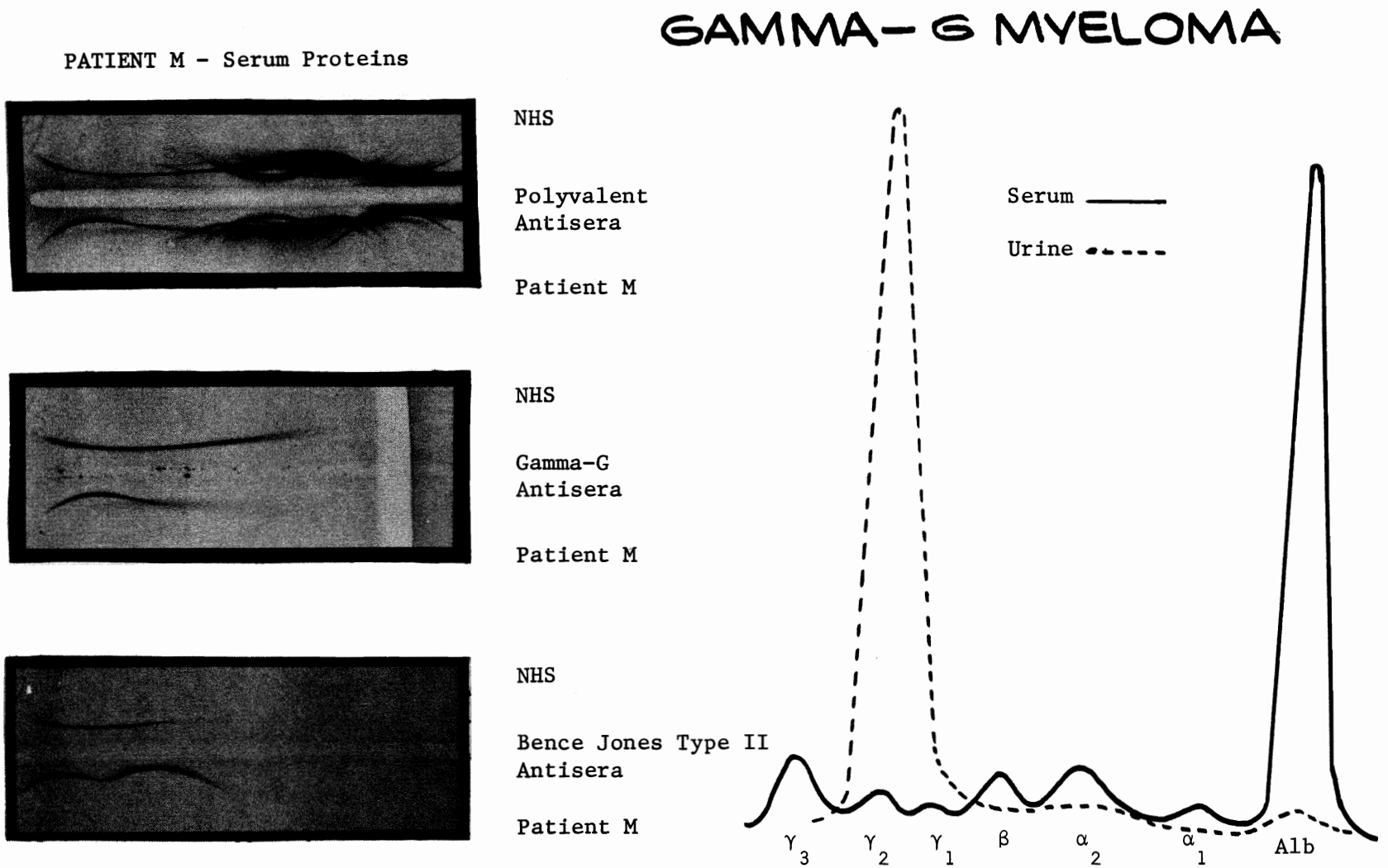
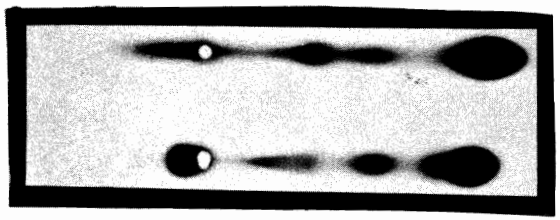


Figure 10. Electrophoretic and immunoelectrophoretic patterns of serum and urine proteins. A normal human serum pattern, designated "NHS", is shown at the top of the immunoelectrophoresis for comparison. The antisera used in the immunoelectrophoresis is indicated to the right of the photograph.

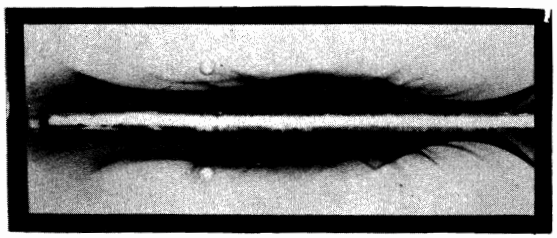
PATIENT S,- Serum Proteins



NHS

Agar-gel
Electrophoresis

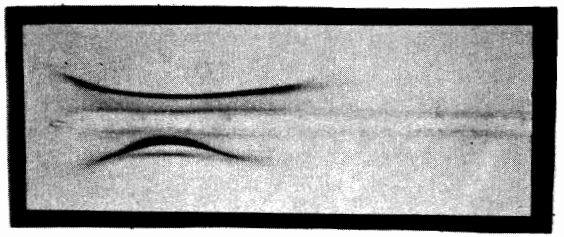
Patient S



NHS

Polyvalent
Antisera

Patient S



NHS

Gamma-G
Antisera

Patient S

GAMMA-G MYELOMA

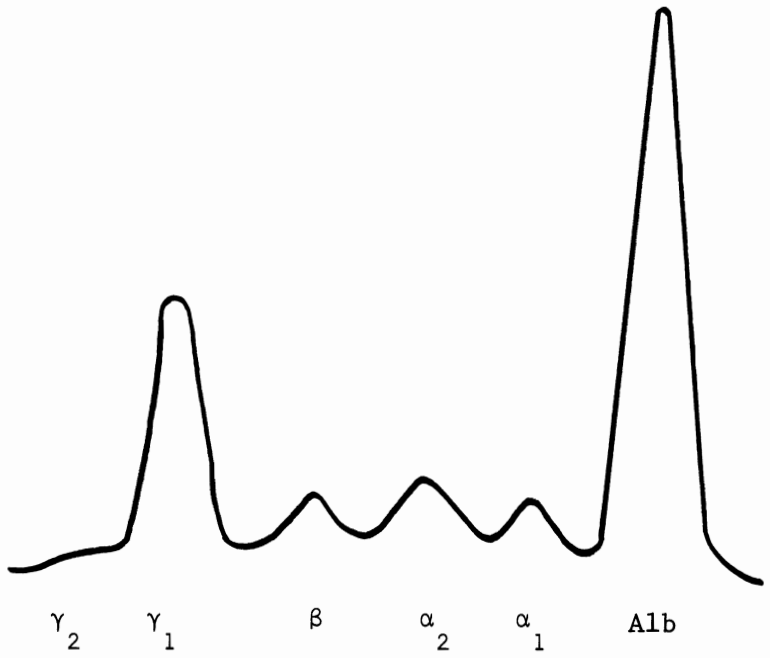


Figure 11. Electrophoretic and immunoelectrophoretic patterns of the serum proteins. A normal human serum pattern, designated "NHS", is shown at the top of the immunoelectrophoresis for comparison. The antisera used in the immunoelectrophoresis is indicated to the right of the photograph.

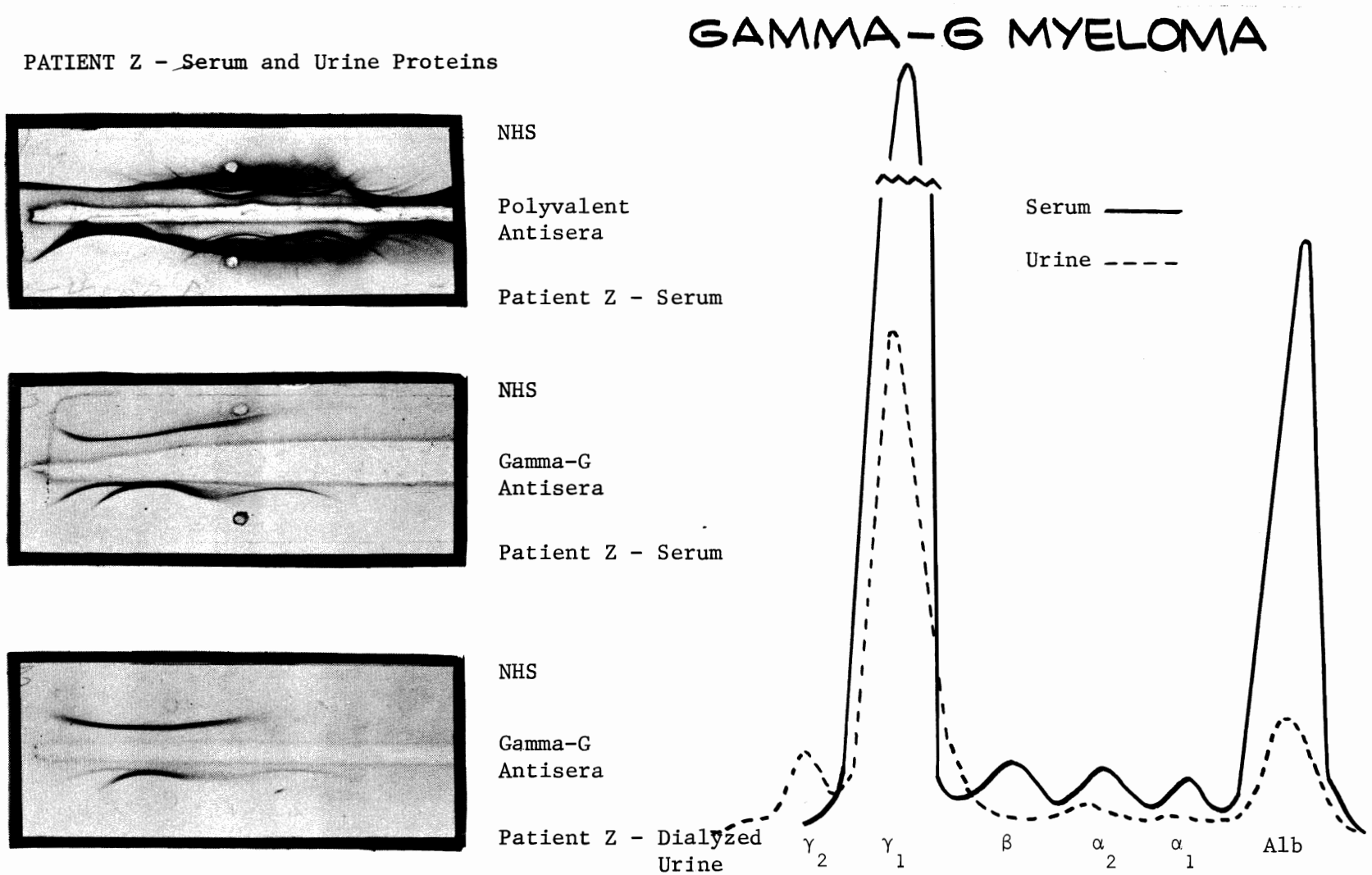


Figure 12. Electrophoretic and immunoelectrophoretic patterns of serum and urine proteins. A normal human serum pattern, designated "NHS", is shown at the top of the immunoelectrophoresis for comparison. The antisera used in the immunoelectrophoresis is indicated to the right of the photograph.

between the gamma and beta globulin regions (see Table 7). The mobility of the M-protein in the urines did not necessarily correlate with the mobility of the M-protein in the sera. Representative examples of the electrophoretic patterns obtained in the Gamma-A myelomas are given in Figure 7 and Figure 8.

In this myeloma series there were two Gamma-D myelomas. No detectable abnormal protein band was evident in the serum or urine of the one case, patient J, while in the other case, Patient K, a very diffuse band was demonstrated in the Gamma-1 region of the serum electropherogram. An abnormal protein peak comprising 90% of the total protein was also seen in the Gamma-1 position in the urine electropherogram (see Figure 9).

Electrophoretically, the Gamma-G myelomatoses constituted a very heterogeneous population in terms of the mobility range of the M-component, the quantity of this component in the serum and urine, and the number of molecular abnormalities expressed in the electrophoretic pattern as additional peaks. Because of the marked heterogeneity in the electrophoretic patterns of the Gamma-G myelomatoses, three cases were selected to demonstrate some of these abnormalities (see Figures 10, 11, and 12). The electropherogram of patient M (see Figure 10) shows three peaks in a region where only one peak is "normally" seen in the serum. A single gamma spike was observed in the urine. Patient S (see Figure 11) had a normal total protein concentration. The electrophoretic fractionation of the serum proteins was quantitatively normal, with the exception of a reduction of the albumin

fraction. While the globulin fractions were present in normal concentrations, an abnormal spike appeared in the gamma region with an apparent reduction of normal gamma globulin. A urine specimen was not obtained from this patient. The serum and urine electrophoretic patterns of patient Z (see Figure 12) represent protein patterns that are frequently associated with so-called "classical" Gamma-G myeloma. The broken line in the gamma globulin fraction indicates that the elevation of the M-component was so marked that the pattern would have exceeded the margins of this chart.

Attempts have been made to distinguish Gamma-A and Gamma-G myelomas according to the differences in the size of the base of the M-protein in the electrophoretic pattern. Laurell and Heremans (1961) have proposed that statistically Gamma-A type myelomas are found to have broader bases than Gamma-G type myelomas. In the present study, this finding was supported in all of the Gamma-A myelomas and in all but two cases of Gamma-G myelomas. In one of the Gamma-G cases, patient P, the paraprotein was associated with a cryoglobulin. In the other case, patient U, the paraprotein appeared electrophoretically as a grossly serrated band in the gamma globulin region on cellulose acetate medium. A serrated band is sometimes produced on cellulose acetate strips when there is a very homogeneous protein in excess. In spite of these findings, this criterion for differentiating myeloma types may not be reliable. First, since the observations of Laurell and Heremans (1961), another myeloma type, Gamma-D, has been reported. Of the two Gamma-D myelomas presented in this report, one

of these cases, patient K, was found to have a diffuse, broad based band (see Figure 9), similar to the Gamma-A type myelomas. A second reason for criticizing the approach of Laurell and Heremans in the prediction of myeloma types is that hypergammaglobulinemias, characterized by a diffuse broad banding of the immunoglobulins on paper or cellulose acetate electrophoresis, may appear in conditions not associated with Gamma-A globulin and not associated with myeloma, such as chronic infection, which is usually associated with elevated levels of Gamma-G globulin or hepatic parenchymal disease, which is frequently associated with Gamma-M globulins (Alper et al., 1966).

III DETECTION OF BENCE JONES PROTEINURIA

With the detection of proteinuria, classification of the anomaly may be relegated into two broad groups: protein disturbances resulting from renal involvement, or protein disturbances of the myelomatous process with an associated Bence Jones proteinuria (Naumann, 1965). Of the many tests that have been devised for the detection of proteinuria, some are too insensitive and others are too nonspecific to be of diagnostic value. Only one or two may be used to differentiate the proteins in the urine.

The indicator test using filter paper strips impregnated with citrate buffered (pH 3.5) tetrabromphenolsulfonphthalein frequently gives false negative reactions in the presence of significant Bence Jones proteinuria due to the pH dependency in the dye reaction (Smith, 1963). The sulfosalicylic acid procedure, a nonspecific

protein test, has been reported to give reactions in the presence of significant amounts of Bence Jones protein, uroglobulins, or albumins (Alper et al., 1966; Naumann, 1965; Smith, 1963). The p-toluene sulfonic acid (TSA) and the heat and acetic acid tests are methods described for the detection of Bence Jones proteinuria. However, it has not always been possible using these tests to distinguish Bence Jones protein from uroglobulins. A protein-paper-extraction test has been devised (Naumann, 1965) which has been reported to be more specific than the two previously mentioned tests and enables differentiation of Bence Jones proteins from the uroproteins. Initial screening tests for Bence Jones proteinuria in this study included the sulfosalicylic acid tests and the Combistix® test. Specific testing was performed with p-toluene sulfonic acid, the heat and acetic acid and the protein-paper-extraction methods. The results of these examinations are given in Table 8.

IV CALCIUM LEVELS AND RADIOLOGIC SURVEYS

Hypercalcemia is purported to be a symptom commonly associated with the widespread skeletal lesions and degenerative changes that occur in the myelomatous process (Osserman, 1965; Brodsky, et al., 1967). Hypercalcemia of multiple myeloma may presumably result from an increase in diffusible calcium due to bone demineralization. Additionally, it is possible that elevated serum calcium levels could be initiated by hyperproteinemia (Rawson and Sunderman, 1948). There are several factors involved in the hyperproteinemia of myeloma that

TABLE 8

DETECTION OF PROTEINURIA

Myeloma Type and Patient Designation	Combistix(R) Test	Sulfosalicylic Acid Test	p-Toluene Sulfonic Acid Test	Heat and Acetic Acid Test	Paper-Extraction Test
Normoproteinemic					
Patient A	pos	---	---	neg	---
Patient B	---	---	---	---	---
Patient C	pos	---	---	neg	---
Patient D	neg	pos	pos	pos	---
Patient E	pos	pos	---	pos	---
Gamma-A					
Patient F	---	pos	---	neg	---
Patient G	pos	---	---	pos	---
Patient H	---	---	pos	pos	pos
Patient I	pos	---	pos	pos	---
Gamma-D					
Patient J	pos	pos	pos	pos	---
Patient K	pos	pos	neg	pos	pos

TABLE 8 - Continued

DETECTION OF PROTEINURIA

Myeloma Type and Patient Designation	Combistix® Test	Sulfosalicylic Acid Test	p-Toluene Sulfonic Acid Test	Heat and Acetic Acid Test	Paper Extraction Test
Gamma-G					
Patient L	pos	---	---	---	pos
Patient M	pos	pos	pos	pos	---
Patient N	pos	---	---	neg	pos
Patient O	pos	pos	---	neg	pos
Patient P	neg	---	---	neg	---
Patient Q	---	---	---	---	---
Patient R	neg	---	---	neg	pos
Patient S	neg	---	---	---	---
Patient T	pos	---	neg	neg	---
Patient U	---	---	---	---	---
Patient V	neg	---	---	neg	---
Patient W	pos	pos	neg	neg	---
Patient X	pos	---	---	neg	---
Patient Y	---	---	---	neg	---
Patient Z	pos	---	---	pos	pos

could account for hypercalcemia. Only nonprotein bound calcium is filtered at the glomerulus (Goldman, 1962) and increased calcium-binding properties of the serum proteins have been reported in myelomatous conditions (Rawson and Sunderman, 1948). However, hyperproteinemia may not be the mechanism involved since there is frequently no correlation between the total serum calcium and the total serum proteins in this disease (Gutman et al., 1936; Aponte, 1963). The data presented herein suggested that hypercalcemia was not necessarily a concurrent finding with osteolysis and bone destruction. The calcium levels and the results of the radiologic surveys, i.e., the presence of osteolysis or pathologic fractures, are shown in Table 9. Of interest is that at the time of diagnosis only three cases exceeded the normal range for the serum calcium concentrations, and with subsequent testing only five cases were found to have increased calcium levels; extensive bony involvement was a usual finding in the myelomatoses studied.

V HEMATOLOGIC FINDINGS

Table 10 lists the hematologic findings at the time of diagnosis of the myelomatoses. In general, these data were consistent with other values reported for the myelomatous conditions. The percentage of plasma cells in the bone marrow ranged from 6% to 80%. In several instances, the plasma cells were not enumerated since the aspirate consisted almost entirely of plasma cells. The low plasma cell percentages reported in other cases may not be representative of the

TABLE 9

SERUM CALCIUM LEVEL AND BONE DEGENERATION

Myeloma Type and Patient Designation	Patient Age	Serum Calcium mg%	Bony Involvement As Evidenced By Radiologic Survey: Osteoporosis	Bony Involvement As Evidenced By Radiologic Survey: Pathologic Fractures
Normoproteinemic				
Patient A	70	9.8	+	+
Patient B		10.4	+	-
Patient C	63	10.3	+	-
Patient D	48	10.3	-	-
Patient E	74	15.6	±	-
Gamma-A				
Patient F	55	10.2	+	+
Patient G	78	10.2	+	+
Patient H	66	8.2	-	-
Patient I	70	12.8		
Gamma-D				
Patient J	44	10.3	-	+
Patient K	62	9.0	+	+

TABLE 9--Continued

Myeloma Type and Patient Designation	Patient Age	Bony Involvement As Evidenced By Radiologic Survey: Serum Calcium mg%	Osteoporosis	Pathologic Fractures
Gamma-G				
Patient L	49	14.2	+	+
Patient M	73	7.2	+	+
Patient N	72	10.0	+	-
Patient O	64	10.8	+	-
Patient P	47	9.0	-	-
Patient Q	63	9.4	+	-
Patient R	72	10.8		
Patient S	61	--	+	+
Patient T	59	9.1	+	-
Patient U	64	9.3	-	+
Patient V	71	10.0	+	+
Patient W	74	--	-	-
Patient X	49	--	-	-
Patient Y	32	10.7	+	+
Patient Z	65	9.8	+	+

TABLE 10

HEMATOLOGICAL FINDINGS AT THE TIME OF DIAGNOSIS

Myeloma Type and Patient Designation	VPRC* (%)	WBC* (mm^3)	Platelets (mm^3)	ESR* (mm/hr)	Rouleaux Formation	%Plasma Cells (Bone Marrow)
Normoproteinemic						
Patient A	40	4,200	Adequate	---	-	31
Patient B	40	5,500	106,000	---	-	15
Patient C	45	5,750	550,000	39	-	Numerous
Patient D	24	11,600	Adequate	---	-	24
Patient E	37	22,700	66,000	---	++	61
Gamma-A						
Patient F	28.5	3,400	138,000	---	++++	42
Patient G	32	7,100	Adequate	60	+	6
Patient H	31	15,200	Decreased	---	-	80
Patient I	27	9,000	178,000	---	+	34
Gamma-D						
Patient J	44	7,400	255,000	36	-	13
Patient K	27	5,200	74,000	---	-	Numerous

TABLE 10-Continued

HEMATOLOGICAL FINDINGS AT THE TIME OF DIAGNOSIS

Myeloma Type and Patient Designation	VPRC* (%)	WBC* (mm^3)	Platelets (mm^3)	ESR* (mm/hr)	Rouleaux Formation	%Plasma Cells (Bone Marrow)
Gamma-G						
Patient L	25	10,400	152,000	68	++++	38
Patient M	27	1,700	52,000	---	+++	--
Patient N	27	3,400	Adequate	66	+++	53
Patient O	34	8,000	Adequate	---	++++	Numerous
Patient P	27	6,200	248,000	61	++	--
Patient Q	40	4,500	226,000	46	-	14
Patient R	31	18,400	Decreased	61	+	76
Patient S	46	5,200	144,000	---	-	16
Patient T	35	4,300	132,000	---	+	6
Patient U	48.5	6,500	Adequate	---	-	Hypoplastic
Patient V	29	5,700	250,000	---	++++	12
Patient W	29.5	7,300	Adequate	54	-	35
Patient X	46	10,000	Adequate	---	-	Normal
Patient Y	45	4,200	---	---	++++	33
Patient Z	32	2,500	122,000	---	++++	--

* VPRC = Volume Packed Red Cells
WBC = White Blood Cells
ESR = Erythrocyte Sedimentation Rate (Wintrobe)

actual proliferation of these cells. Frequently at autopsy a massive plasma cell infiltration throughout the bone marrow and reticulo-endothelial organs was found even though puncture or biopsy may not have shown bone marrow infiltration of plasma cells early in the course of the disease.

VI IMMUNOLOGIC CHARACTERIZATION AND QUANTITATION

The application of the principles of immunoelectrophoresis enables classification of the myeloma proteins into a number of composite globulin groups (see Table 4). These immunoglobulins may be found to exhibit many different molecular aberrations. For example, Figure 10 illustrates a serum electrophoretic pattern with three gamma globulin moieties, yet the abnormality falls into the Gamma-G group because the serum contains an increase of a very homogeneous Gamma-G globulin with an L-chain subunit. The moiety labeled as Gamma-1 (Figure 10) was presumed to be normal Gamma-A and Gamma-M globulin in reduced concentrations. The moiety labeled Gamma-2 consisted of lambda-type (designated Type II in Figure 10) light chains unassociated with any heavy chains of gamma globulin. The moiety labeled as Gamma-3 consisted of Gamma-G globulin heavy chains associated with lambda-type light chains. The urine consisted predominantly of unassociated lambda-type Bence Jones proteins. In addition to these molecular aberrations, polymerization may occur (Bernier and Putnam, 1963). Light chains are frequently dimerized. If the paraprotein in the serum is found to consist of a homogeneous

immunoglobulin molecule, the urine may contain only one of the polypeptide components, either as a monomer, dimer or polymer.

The use of specific heavy chain antisera has allowed the classification of the myelomatoses into the Gamma-G, Gamma-A, or Gamma-D groups (see Figure 7 through Figure 12). Because of the considerable overlap of the mobility of the Gamma-A, Gamma-D, Gamma-G and Gamma-M globulins upon paper or cellulose acetate electrophoresis, serologic identification of the immunoglobulin is necessary to distinguish these groups. Figure 7 illustrates the electrophoretic patterns of the serum and urinary proteins from a Gamma-A myeloma. The serum abnormality is in the beta region while the urine abnormality is in the gamma region. Since both Gamma-G and Gamma-A paraproteins may occupy these electrophoretic regions, without serologic identification, classification would be impossible. Immunoelectrophoresis characterized both of these proteins as Gamma-A globulins.

The urine of normoproteinemic myelomas usually contain an abnormal protein which does not consist of any of the common gamma immunoglobulin heavy chains (IgG, IgA, IgM, or IgD). Figure 6 gives an example of this nonreactivity with polyvalent antisera. The urine appears to be devoid of any of the immunoglobulin components, yet strong reactivity is obtained with specific light chain antisera.

A classification of each of the myeloma groups was made on the basis of reactivity with type specific light chain antisera. Each of the immunoglobulin groups in normal human sera contain both kappa and

lambda type light chains with an approximate frequency of 2:1 respectively (Mannik and Kunkel, 1963b). The myeloma proteins contain only one type of light chain polypeptide.. In most instances, the light chain antigenic typing of the myeloma protein in the serum corresponds to the Bence Jones protein excreted in the urine (Mannik and Kunkel, 1962). In this series the serum or urinary proteins of ten patients reacted with kappa light chain antisera and the serum or urinary proteins of an additional ten patients reacted with lambda light chain antisera. Table 11 lists the kappa and lambda light chain typing and immunoglobulin quantitation of the Gamma-A, Gamma-G, and Gamma-M proteins of the patients in this series.

Of particular interest in this study was the observation that when the IgA and IgD myeloma proteins were found to consist of lambda type light chains there was a concommitant reduction of IgG immunoglobulin concentration (see Table 11). Conversely, when the light polypeptide chains of the IgA and IgD myeloma proteins reacted with kappa type light chain antisera Gamma-G levels were quantitatively normal.

TABLE 11

LIGHT CHAIN DESIGNATION AND IMMUNOGLOBULIN CONCENTRATION

Myeloma Type and Patient Designation	Specific Light Chain	IMMUNOGLOBULIN QUANTITATION (mg/100 ml) Serum Level IgG	IgA	IgM	Urine Level IgG	IgA	IgM
Normal Range (± 1 S.D.)		1020-1460	300-480	85-155	Unknown	Unknown	Unknown
Normoproteinemic							
Patient A	Kappa	---	--	--	---	--	-
Patient B	Kappa	320	95	20	120	0	0
Patient C	-----	---	--	--	---	--	-
Patient D	-----	650	40	25	210	20	15
Patient E	Lambda	1050	50	40	300	30	0
Gamma-A							
Patient F	Kappa	1050	4000	30	100	40	0
Patient G	Kappa	1200	620	30	270	300	0
Patient H	Lambda	205	490	15	10	225	0
Patient I	Lambda	350	2100	0	---	--	-
Gamma-D							
Patient J	Kappa	1250	70	15	---	--	-
Patient K	Lambda	240	40	15	125	0	0

TABLE 11--Continued

LIGHT CHAIN DESIGNATION AND IMMUNOGLOBULIN CONCENTRATION

Myeloma Type and Patient Designation	Specific Light Chain	IMMUNOGLOBULIN QUANTITATION (mg/100 ml) Serum Level IgG	IgA	IgM	Urine Level IgG	IgA	IgM
Gamma-G							
Patient L	Kappa	2040	40	30	1500	0	0
Patient M	Lambda	1500	45	20	125	0	0
Patient N	Kappa	2630	35	15	600	0	0
Patient O	Lambda	950	45	20	400	0	0
Patient P	Kappa	1350	45	35	175	0	0
Patient Q	Kappa	1910	50	50	290	25	0
Patient R	Lambda	2060	35	10	840	0	0
Patient S	------	1920	55	30	---	-	-
Patient T	Lambda	1970	55	20	840	20	0
Patient U	------	----	--	--	---	-	-
Patient V	Lambda	----	--	--	---	-	-
Patient W	------	4400	950	20	0	40	0
Patient X	Lambda	----	--	--	215	0	0
Patient Y	------	1850	50	30	25	0	0
Patient Z	Kappa	1875	35	20	775	25	0

DISCUSSION

Immunologic and physiochemical procedures have been employed in the study of twenty-six selected cases of multiple myeloma. These plasmacytic dyscrasias include examples of M-protein elaboration of the Gamma-A, Gamma-D, and Gamma-G immunoglobulins or their polypeptide subunits. The majority of these cases had a single abnormal component in the serum. In the instances where a paraprotein was present in the urine, this component appeared to be of the same light chain type of immunoglobulin class as the serum component. Also included in this study were examples of the Bence Jones or normoproteinemic myelomas. An apparent disorder in normoproteinemic myelomas is not readily discernible in the serum, but myelomatous disease becomes evident from the proliferation of plasma cells, skeletal degeneration and the frequent occurrence of an electrophoretically homogeneous urinary protein.

The results of detailed analyses of the serum and urinary protein characteristics and other relevant laboratory findings of each of the twenty-six myeloma cases studied has been presented in tabular form. Table 6 through Table 11 should be consulted for the specific laboratory data of any single case. Only representative electrophoretic and immunoelectrophoretic patterns from each of the gammopathies studied has been presented in Figure 6 through Figure 12.

The Gamma-G and normoproteinemic myelomas are of particular interest from the standpoint of molecular heterogeneity. The proteins from these groups represent a large heterogeneous population

of antibodies with different specificities. The Gamma-A myelomas appear to be the most homogeneous group by a variety of criteria (Franklin and Lowenstein, 1964). The Gamma-D myelomas will in all probability, also, be distinguished by their homogeneity, since these proteins have been selected out of the normoproteinemic class.

Doubtlessly, the normoproteinemic group will yield other new classes of immunoglobulins, but description must await proliferative changes resulting in a copious production of an antibody that would "normally" be present in trace quantities. In order to form a new immunoglobulin class, the antisera to the protein must be nonreactive with the proteins of the established immunoglobulin classes (W.H.O. 1964; Kunkel et al., 1966) and should presumably react at least minimally with a component present in normal human sera.

The largest and perhaps most heterogeneous class of immunoglobulins is the Gamma-G proteins. Among other activities, the antibodies in this class effect the neutralization of viruses and bacterial toxins (Gitlin, 1966). The proteins in this class are related immunochemically, but also contain many distinct components as evidenced by the serum electrophoresis of Patient M and the immunoelectrophoresis of Patient Z.

In general, hyperproteinemia was not the characteristic finding in the myelomatoses studied; half of the patients in this series were found to be normoproteinemic or hypoproteinemic. Erythrocyte sedimentation rates and rouleaux formation have been used to indicate the presence of these serum immunoglobulin abnormalities (Marmont,

et al., 1957; Levin and Ritzmann, 1966). The expected correlation of elevated serum calcium levels with the occurrence of osteoporosis and skeletal degeneration was not evident. Bone destruction as a result of the myelomatous process was indicated by radiologic survey in 19 of the twenty-six patients; whereas, hypercalcemia was present in only five patients.

The chemotherapeutic agent, Melphalan, has been reported to be of value in the management of the myelomas, with a preferential response occurring in kappa type myelomas (Bergsagel, et al., 1965). Light chain typing was undertaken and the clinical course of the disease was reviewed, however insufficient follow-up studies precluded any evaluation of the therapeutic response in these patients.

The correlation of immunocyte (plasma cells and lymphocytes) proliferation with immunoglobulin production has been described by Bjoerneboe and Gormsen (1943), Fagraeus (1948), and Campbell and Good (1949). Of the cases studied herein the probable source of the abnormal human immunoglobulins in multiple myeloma was the plasma cell. Marked plasma cell proliferation was evident from bone marrow aspirate or histologic studies at autopsy.

In general, the class specific immunoglobulin antisera used in this study strongly demarcated the M components into each of the immunoglobulin classes; however, light chain differentiation was not so pronounced. The reliability of immunologic typing with antisera is questionable because of the procedure involved in the preparation of this material. This is particularly evident in the preparation of

light chain antisera. The most apparent inconsistency is introduced by the antigen used to elicit antibody response. The most available sources of antigen are the pathologic proteins produced in myelomatous conditions. Amino acid sequence analyses of these proteins have revealed that for each individual case of multiple myeloma a distinct kappa or lambda chain is produced; therefore, the antisera from a single paraprotein would not be expected to react with all other proteins within that specific light chain type.

Amino acid residue studies have shown that the peptide sequences of the C terminal half of light chains are identical, whereas the N terminal half of the paptide has considerable variation (Hilschmann and Craig, 1965; Titani et al., 1965). It is a likely supposition that the kappa and lambda antigenic determinants may be present on the common portion of the light chain, while the unique reactivity of the light chain in myelomatoses may be accounted for in the variable portion of the polypeptide (Fleischman, 1966).

An additional inherent difficulty in the preparation of light chain antisera is the presence of extraneous protein material in the antigenic source. Since concentrated urine proteins are frequently used as the antigenic source, proteins other than the immunoglobulins are present. Other proteins present in trace amounts are principally albumin, transferrin and urine mucoids.

Type specific reactivity of light chain antisera requires purification of the antisera. The purification method introduces several other inconsistencies. Type specificity is produced by

absorption of the antisera with the heterologous light chain type. The antigen used for this absorption is also obtained from pathologic material and has the same margin for error as was previously discussed. A second inconsistency is introduced by the dissociation of the antigen-antibody complex during the absorption process. The 7S antigen complexes are soluble in antigen excess, therefore, incomplete absorption of the heterologous light chain antibody may be obtained.

Quantitative measurements of the normal immunoglobulins in plasmacytic malignancies indicate that there is a reduction in the synthesis of these proteins (Barth et al., 1964; Solomon et al., 1963b). It has been reported that in multiple myeloma the abnormal immunoglobulin is greatly increased, while all other gamma globulin components are markedly reduced (Fahey and Lawrence, 1963). It was observed in this study that there was a direct correlation between the specific light chain type of the Gamma-A and Gamma-D myeloma proteins and the quantity of the Gamma-G protein elaborated. Kappa type Gamma-A and Gamma-D myelomas were associated with normal levels of Gamma-G, while lambda type Gamma-A and Gamma-D myelomas were associated with a reduced level of Gamma-G protein. This relationship was not evident in the normoproteinemic myelomas. The Gamma-G levels of the Gamma-A and Gamma-D myelomas reported here are not consistent with the reduced immunoglobulin levels referred to above. A confirmation of these findings would necessitate the study of larger numbers of these two gammopathies.

It has been suggested that with the occurrence of immunocyte differentiation an individual cell is confined to the production of one type of class specific polypeptide (α, δ, γ or μ) and one type of light polypeptide (κ or λ) (Curtain et al.,1959; van Furth, 1964; Bernier and Cebra, 1965). With rare exception, more than one type of heavy chain does not appear within the same cell, nor are both kappa and lambda polypeptide chains found coexisting within the single cell. However, most individual cells contain both of these molecular species, i.e., one class of heavy polypeptide and one type of light polypeptide. Some cells have been found to contain only one molecular species, either one type of light chain or one class of heavy chain, with the concomitant absence of the other constitutive polypeptide of the antibody molecule (Bernier and Cebra, 1965). Therefore, it would be expected that cells exist which are exclusive producers of a single molecular species.

Since lambda type polypeptides are produced with less frequency than the kappa type proteins it is plausible that the limiting factor in lambda type myeloma could be the availability of these polypeptides. This concept would be particularly feasible in the Gamma-A and the Gamma-D myelomas, because the Gamma-A and Gamma-D proteins "normally" represent only a small portion of the total serum immunoglobulin level (21% and 0.2%, respectively, Fahey, 1965). In neoplastic states these immunoglobulins must have a high rate of synthesis in order to account for the serum levels obtained,

especially if their catabolic rates are increased in hypergammaglobulinemic states, as are the catabolic rates of Gamma-G in conditions of excessive production. Apparently it is possible for the catabolic rates of Gamma-G globulin to double or triple if serum protein levels are greatly increased (Fahey and Robinson, 1963).

A number of mechanisms could exist to account for the reduction of Gamma-G components in the lambda type myelomas on the basis of cellular synthesis or the utilization of catabolic products. If immunocyte differentiation did not occur or if mature immunocytes were found to produce more than one distinct immunoglobulin, an explanation would be apparent. However, if lambda type light chains were the limiting factor in Gamma-G production, it would be anticipated that in Gamma-A and Gamma-D myelomas assays for free serum lambda chains should be negative. The assays of Williams et al., (1966) do not confirm this hypothesis. It is suggested that another approach to this problem might be through the use of immunofluorescent localization studies of the immunoglobulins in the plasma cells of the bone marrow. It is possible that this procedure would ascertain whether or not plasma cell differentiation actually occurs in the malignant states.

SUMMARY

1. Serum and urinary proteins from 26 patients with myeloma were analyzed serologically and electrophoretically. On the basis of such analyses the 26 cases were classed serologically as Gamma-A, Gamma-D, Gamma-G and normoproteinemic.
2. Hematological, radiological, and other clinical findings associated with the myelomatous disease, or otherwise applicable to interpretative analyses of serological data, were reviewed.
3. In general, hyperproteinemia was not the usual finding in the gammopathies studied. Total serum protein values of less than 8.0 gm/100 ml occurred in the sera of 13 cases in this series.
4. Considerable diversification was present in the electrophoretic patterns of the serum and urinary proteins. The evidence of an abnormal component ranged from negligible to the appearance of three distinct peaks in an electrophoretic region where only one peak "normally" occurs. Quantitative estimation of an M component in the sera ranged from negligible to 8.8 gm/100 ml.
5. Proteinuria was detected by the sulfosalicylic acid test, the Combistix® test, the p-toluene sulfonic acid test, the heat and acetic acid test, and the protein-paper-extraction test. The presence of proteinuria was demonstrated in the urine of 18 of the 23 patients tested.
6. Hypercalcemia was not a common finding in this study, while

extensive bony involvement was a usual finding. Evidence of lytic skeletal destruction was found in 19 patients; associated hypercalcemia was apparent in only 2 of these patients.

7. Hematologic findings did not differ appreciably from other values reported for the myelomatoses.

8. Proteins of 20 patients were typed with light chain antisera. Of these, 50% were found to be kappa type light chains and 50% were lambda type light chains. In this series Gamma-A and Gamma-D myeloma proteins consisting of lambda type light chains were associated with a reduced serum level of IgG immunoglobulin, whereas Gamma-A and Gamma-D proteins consisting of kappa type light chains were associated with quantitatively normal serum IgG levels.

BIBLIOGRAPHY

Alper, C.A., Rosen, F.S. and Janeway, C.A. 1966. The gamma globulins, II. Hypergammaglobulinemia. New Engl. J. Med. 275: 591-596.

Apitz, K. 1940. Die paraproteinosen. (Uber die storung des eiweisstoffwechsels bei plasmocytom.) Virchows Arch. Path Anat. 306: 631-699.

Aponte, G.E. 1963. Nephrocalcinosis and nephrolithiasis in hyperparathyroidism. In: Sunderman, F.W. and Sunderman, F.W. Jr. (eds.) Evaluation of Thyroid and Parathyroid Functions. J.B. Lippincott Co. Philadelphia. pp. 263-264.

Askonas, B.A. and Rhodes, J.M. 1965. Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. Nature 205: 470-474.

Asofsky, R. and Thorbecke, G.J. 1961. Sites of formation of immune globulins and of a component of C'3: II. Production of immunoelectrophoretically identified serum proteins by human and monkey tissue in vitro. J. Exp. Med. 114: 471-483.

Augustin, R. and Hayward, B.J. 1960. Cleavage of human γ-globulin. Nature 187: 129-130.

Baglioni, C., Zonta, L.A., Cioli, D. and Carbonara, A. 1966. Allelic antigenic factor inv (a) of the light chains of human immunoglobulins: Chemical basis. Science 152: 1517-1518.

Barth, W.F., Wochner, T.A. and Fahey, J.L. 1964. Metabolism of human gamma macroglobulins. J. Clin. Invest. 42: 1036-1048.

Bennett, J.C., Hood, L., Dreyer, W.J. and Potter, M. 1965. Evidence for amino acid sequence differences among proteins resembling the L-chain subunits of immunoglobulins. J. Mol. Biol. 12: 81-87.

Berggard, I. 1961. Studies on the plasma proteins in normal human urine. Clin. Chim. Acta 6: 413-429.

Bergsagel, D.E., Migliore, P.J. and Griffith, K.M. 1965. Myeloma proteins and the clinical response to melphalan therapy. Science 148: 376-377.

Bernier, G.M. and Cebra, J.J. 1964. Polypeptide chains of human gammaglobulin: Cellular localization by fluorescent antibody. Science 144: 1590-1591.

Bernier, G.M. and Cebra, J.J. 1965. Frequency distribution of α, γ, κ and λ polypeptide chains in human lymphoid tissues. J. Immunol. 95: 246-253.

Bernier, G.M. and Putnam, F.W. 1963. Monomer dimer form of Bence Jones proteins. Nature 200: 223-225.

Bjerneboe, M. and Gormsen, H. 1943. Experimental studies on the role of plasma cells as antibody producers. Acta Path. Microbiol. Scand. 20: 649-692.

Boyd, W.C. and Bernard, H. 1937. Quantitative changes in antibodies and globulin fractions in sera of rabbits injected with several antigens. J. Immunol. 33: 11-122.

Brodsky, I., Kahn, S.B. and Meyer, A.N. 1967. Treatment of multiple myeloma. Geriatrics 22: 140-148.

Buckley, C.E., Whitney, P.L. and Tanford, C. 1963. The unfolding and renaturation of a specific univalent antibody fragment. Proc. Natl. Acad. Sci. U.S. 50: 827-834.

Burckhardt, A.E. 1883. Beitrage zur chemie und physiologie des blutserums. Arch. Pathol. Pharmakol. 16: 322-343.

Burnet, F.M. 1957. A modification of Jerne's theory of antibody production using the concept of clonal selection. Australian J. Sci. 20: 67.

Burnet, F.M. 1959. The Clonal Selection Theory of Acquired Immunity. Vanderbilt Univ. Press, Nashville, Tenn.

Burtin, P. and Buffe, D. 1963. Immunofluorescent studies of human plasma cells in γ and B_2A myelomas. Proc. Soc. Exp. Biol. Med. 114: 171-175.

Burtin, P., Hartmann, L., Fauvert, R. and Gravar, P. 1956. Proteins of myeloma. I. Critical study of technics of identification of the Bence Jones protein and their diagnostic value. Rev. Franc. Etudes Clin. Biol. 1: 17-28.

Butler, A.M. and Montgomery, H.J. 1933. Solubility of plasma proteins; dependence on salt and plasma concentrations in concentrated solutions of potassium phosphate. J. Biol. Chem. 99: 173-195.

Campbell, D.H., Garvey, J.S., Cremer, N.S. and Sussdorf, N.S. 1963. Methods In Immunology. W.A. Benjamin, Inc. New York.

Carbonara, A.O., Rodhain, J.A. and Heremans, J.I. 1963. Localization of γ 1_A-globulin (B_{2A}-globulin) in tissue cells. Nature 198: 999-1000.

Cawley, L.P. 1966. Workshop manual on electrophoresis and immuno-electrophoresis, Commission on Continuing Education. Am. Soc. Clin. Pathol., Chicago, Ill.

Cohen, E. and Raducha, J.J. 1962. Detection of urinary Bence Jones protein by means of p-toluene sulfonic acid (T.S.A.) Am. J. Clin. Pathol. 37: 660-663.

Cohen, S. 1963a. Properties of the separated chains of human γ-globulin. Nature 197: 253-255.

Cohen, S. 1963b. Properties of the peptide chains of normal and pathological human gamma globulins. Biochem. J. 89: 334-341.

Cohen, S. and Porter, R.R. 1964. Structure and biological activity of immunoglobulins. Advan. Immunol. 4: 287-349.

Cohn, E.J., McMeeckin, T.L., Oncley, J.L., Newell, J.M. and Hughes, W.L. Proteins. I. Size and charge of proteins separating upon equilibrium across membranes with ammonium sulfate solutions of controlled pH, ionic strength and temperature. J. Am. Chem. Soc. 62: 3386-3393.

Cohn, E.J. and Edsall, J.R. 1943. Proteins, Amino Acids and Peptides. Reinhold, New York, pp. 586-622.

Crowle, A.J. 1961. Immunodiffusion. Academic Press, New York.

Curtain, C.C. and O'Dea, J.F. 1959. Possible sites of macroglobulin synthesis: A study made with fluorescent antibody. Aust. Ann. Med. 8: 143-150.

Denis, P.S. 1859. Memoire sur le Sang., Paris. Cited by Tullis, J.L. (ed.) 1953. Blood Cells and Plasma Proteins, Academic Press Inc., New York. p. 13.

Deutsch, H.F. and Nichol, J.C. 1948. Biophysical studies of blood plasma proteins; Fractionation studies of normal and immune horse serum. J. Biol. Chem. 176: 797-812.

Dray, S. 1960. Three γ-globulins in normal human serum revealed by monkey precipitons. Science 132: 1313-1314.

Dreyer, W.J. and Bennett, J.C. 1965. The molecular basis of antibody formation: A paradox. Proc. Natl. Acad. Sci. U.S. 54: 864-869.

Edelman, G.M. 1959. Dissociation of γ-globulin. J. Am. Chem. Soc. 81: 3155-3156.

Edelman, G.M., Benaceraf, B., Ovary, Z. and Poulik, M.D. 1961. Structural differences among antibodies of different specificities. Proc. Natl. Acad. Sci. U.S. 47: 1751.

Edelman, G.M. and Gally, J.A. 1962. Nature of Bence Jones proteins: Chemical similarities to polypeptide chains of myeloma globulins and normal γ-globulins. J. Exp. Med. 116: 207-227.

Edelman, G.M. and Gally, J.A. 1964. A model for the 7S antibody molecule. Proc. Natl. Acad. Sci. U.S. 51: 846-853.

Edelman, G.M., Heremans, J.F., Heremans, M.T. and Kunkel, H.G. 1960. Immunological studies of human γ-globulin: Relation of the precipitin lines of whole γ-globulin to those of the fragments produced by papain. J. Exp. Med. 112: 203-223.

Edelman, G.M. and Poulik, M.D. 1961. Studies on structural units of the γ-globulins. J. Exp. Med. 113: 861-884.

Epstein, W.V. and Gross, D. 1964. Naturally occurring human antibody reacting with Bence Jones protein. J. Exp. Med. 120: 733-745.

Fagraeus, A. 1948. Antibody production in relation to development of plasma cells: In vivo and in vitro experiments. Acta Med. Scand. (Suppl.) 204: 3-122.

Fahey, J.L. 1962. Evidence for heterogeneity of plasma cells: Studies of proteins produced by plasma cell tumors in inbred mice. Ann. N.Y. Acad. Sci. 101: 221-234.

Fahey, J.L. 1963. Two types of 6.6 S γ-globulins, B_{2A}-globulins and 18S γ_1-macroglobulins in normal serum and γ_1-macroglobulins in normal urine. J. Immunol. 91: 438-459.

Fahey, J.L. 1965. Antibodies and immunoglobulins, I. Structure and function. J. Am. Med. Assoc. 194: 71-74.

Fahey, J.L. and Lawrence, M.E. 1963. Quantitative determination of 6.6S γ-globulins, B_2A-globulins and γ1-macroglobulins in human serum. J. Immunol. 91: 597-603.

Fahey, J.L. and Robinson, A.G. 1963. Factors controlling serum γ-globulin concentration. J. Exp. Med. 118: 845-868.

Fahey, J.L. and Solomon, A. 1963. Two types of γ-myeloma proteins, B_{2A}-myeloma proteins, γ_1-macroglobulins and Bence Jones proteins identified by two groups of common antigenic determinents. J. Clin. Invest. 42: 811-822.

Feinstein, A. and Rowe, A.J. 1965. Molecular mechanism of formation of antigen-antibody complex. Nature 205: 147-149.

Fishman, M. 1961. Antibody formation in vitro. J. Exp. Med. 114: 837-856.

Fishman, M. and Adler, F.L. 1963. Antibody formation initiated in vitro. II. Antibody synthesis in x-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen. J. Exp. Med. 117: 595-602.

Fitch, F.W. and Wissler, R.W. 1965. The histology of antibody production. In: Samter, M. (ed.) Immunological Diseases, Little, Brown and Co., Boston. pp. 65-86.

Fleischman, J.B. 1966. Immunoglobulins. Ann. Rev. Biochem., Part II. 35: 835-872.

Fleischman, J.B., Pain, R.H. and Porter, R.R. 1962. Reduction of γ-globulins. Arch. Biochem. Biophys. (Suppl.) 1: 174-180.

Fleischman, J.B., Porter, R.R. and Press, E.M. 1963. The arrangement of the peptide chains in γ-globulin. Biochem. J. 88: 220-228.

Fougereau, M. and Edelman, G.M. 1965. Corroboration of recent models of the gamma-G immunoglobulin molecule. J. Exp. Med. 121: 373-393.

Fraenkel-Conrat, H. 1963. Chemistry of proteins. In: Florkin, M. and Stotz, E.H. (eds.) Comprehensive Biochemistry, Elsevier, New York. Vol. 7. pp. 56-106.

Franek, F. 1961. Dissociation of animal 7S γ-globulins by cleavage of disulfide bonds. Biochem. Biophys. Res. Commun. 4: 28-32.

Franklin, E.C. 1959. Physicochemical and immunologic studies of γ-globulin of normal human urine. J. Clin. Invest. 38: 2159-2167.

Franklin, E.C. 1960. Structural units of 7S γ-globulin. J. Clin. Invest. 39: 1933-1941.

Franklin, E.C. and Lowenstein, J. 1964. Protein abnormalities associated with proliferative disorders of plasma cells and lymphocytes. Seminars in Hematology 1: 144-164.

Franklin, E.C., Lowenstein, J., Bigelow, B. and Meltzer, M. 1964. Heavy chain disease: A new disorder of serum gamma globulins. Report of the first case. Am. J. Med. 37: 332-350.

Franklin, E.C. and Stanworth, D.R. 1961. Antigenic relationships between immune globulins and certain related paraproteins in man. J. Exp. Med. 114: 521-533.

Frangione, B. and Franklin, E.C. 1965a. Structural studies of human immunoglobulins; Differences in the Fd fragments of the heavy chains of myeloma proteins. J. Exp. Med. 122: 1-10.

Frangione, B. and Franklin, E.C. 1965b. Structural studies of human immunoglobulins. Arch. Biochem. Biophys. 111: 603-613.

Frangione, B., Franklin, E.C., Fudenberg, H.H. and Koshland, M.E. 1966. Structural studies of human gamma-G myeloma proteins of different antigenic subgroups and genetic specificities. J. Exp. Med. 124: 715-732.

Gally, J.A. and Edelman, G.M. 1964. Protein-protein interactions among L polypeptide chains of Bence Jones proteins and human gamma-globulins. J. Exp. Med. 119: 817-836.

Garvey, J.S. and Campbell, D.H. 1957. The retention of S^{35}-labeled bovine serum albumin in normal and immunized rabbit liver tissue. J. Exp. Med. 105: 361-372.

Gitlin, D. 1966. Current aspects of the structure, function and genetics of the immunoglobulins. Ann. Rev. Med. 17: 1-22.

Gitlin, D. and Merler, E. 1961. Comparison of peptides released from related rabbit antibodies by enzymatic hydrolysis. J. Exp. Med. 114: 217-230.

Goldman, R. 1962. Chromic renal failure. In: Clinical Disorders of Fluid and Electrolyte Metabolism. Maxwell, M.H. and Kleeman, C.R. (eds) McGraw-Hill Book Co., New York. p 247.

Grabar, P. and Williams, C.A. 1955. Methode immuno-electrophoretique d'analyse de melanges de substanges antigeniques. Biochim. Biophys. Acta 17: 67-74.

Grey, H.M. and Kunkel, H.G. 1964. H-chain subgroups of myeloma proteins and normal 7S γ-globulin. J. Exp. Med. 120: 253-266.

Grey, H.M., Mannik, M. and Kunkel, H.G. 1965. Individual antigenic specificity of myeloma proteins: Characteristics and localization to subunits. J. Exp. Med. 121: 561-575.

Gutman, A.B. 1948. The plasma proteins in disease. Advan. Protein Chem. 4: 155-250.

Gutman, A.B., Moore, D.H., Gutman, E.B., McCellan, U. and Kabat, E.A. 1941. Fractionation of serum proteins in hyperproteinemia with special reference to multiple myeloma. J. Clin. Invest. 20: 765-783.

Gutman, A.B., Tyson, T.L. and Gutman, E.B. 1936. Serum calcium, inorganic phosphorus and phosphatase activity in hyperparathyroidism, Paget's disease, multiple myeloma and neoplastic disease of the bone. Arch. Int. Med. 57: 379-413.

Gyenes, L., Bata, J.E. and Schon, A.H. 1966. Degradation of rabbit antibodies with papain in presence of urea. Immunochemistry 3: 251-256.

Haber, E. 1964. Recovery of antigenic specificity after denaturation and complete reduction of disulfides in papain fragment of antibody. Proc. Natl. Acad. Sci. U.S. 52: 1099-1106.

Hammarsten, O. 1897. Z. Physiol. Chem. 8: 467. Cited by Haurowitz, F. 1963. The Chemistry and Function of Proteins. 2nd ed. Academic Press, New York. p 184.

Hansson, U.B., Laurell, C.B. and Bauchman, R. 1966. Sedimentation constants of IgG and IgD myeloma proteins with those of normal IgG. Acta Med. Scand. (Suppl.) 445: 89-92.

Hardy, W.B. 1905. Colloidal solution: The globulins. J. Physiol. 33: 251-337.

Hart, J.S., Lawrence, M.C., Ritzmann, S.E. and Levin, W.C. 1965. II. Hyper-and hypoproteinemias 1. Hyperproteinemia correlation of elevated total serum protein values and their responsible globulin fractions with various polyclonal and monoclonal gammopathies: A study of 173 sera. Texas Rep. Biol. Med. 23: 445-457.

Haurowitz, F. 1935. Kolloid-Z. 71: 198. Cited by Haurowitz, F. 1963. The Chemistry and Function of Proteins. Academic Press, New York. p.123.

Haurowitz, F. 1936a. Kolloid-Z. 74: 208. Cited by Haurowitz, F. 1963. The Chemistry and Function of Proteins. Academic Press, New York. p. 123.

Haurowitz, F. 1936b. Kolloid-Z. 77: 65. Cited by Haurowitz, F. 1963. The Chemistry and Function of Proteins. Academic Press, New York. p. 123.

Haurowitz, F. 1942. Separation and determination of multiple antibodies. J. Immunol. 43: 331-340.

Heimer, R. 1966. The Fd-fragment of human γ-immunoglobulin. Immunochemistry 3: 81-85.

Heremans, J.F. 1960. Les Globulines Seriques du Systeme Gamma Editions Aracia, S.A. Brussels.

Herzenberg, L.A. 1964. A chromosome region for $gamma_{2A}$ and $beta_{2A}$ globulin H-chain isoantigens in the mouse. Cold Spring Harbor Symp. Quant. Biol. 29: 455-462.

Hill, R.L. 1965. Hydrolysis of proteins. Advan. Protein Chem. 20: 37-107.

Hill, R.L., Delaney, R., Fellows, R.E. Jr., and Lebovitz, H.F. 1966. The evolutionary origins of the immunoglobulins. Proc. Natl. Acad. Sci. U.S. 56: 1762-1769.

Hilschmann, N. and Craig, L.C. 1965. Amino acid sequence studies with Bence Jones proteins. Proc. Natl. Acad. Sci. U.S. 53: 1403-1409.

Hirschfield, J. 1960. Immunoelectrophoresis: Procedure and application to the study of group-specific variations in sera. Sci. Tools 7: 18-25.

Hobbs, J.R., Slot, G.M.J., Campbell, C.H., Clein, G.P., Scott, J.T., Crowther, D., and Swan, H.T. 1966. Six cases of gamma-D myelomatosis. Lancet 2: 614-618.

Howe, P.E. 1921a. The use of sodium sulfate as the globulin precipitant in the determination of proteins in blood. J. Biol. Chem. 49: 93-107.

Howe, P.E. 1921b. The determination of proteins in blood: A micro method. J. Biol. Chem. 49: 109-113.

Hsiao, S. and Putnam, F.W. 1961. The cleavage of human γ-globulin by papain. J. Biol. Chem. 236: 122-135.

Ishizaka, K., Ishizaka, T., and Hornbook, M.M. 1966. Physicochemical properties of reaginic antibody and correlation of reaginic activity with γE-globulin antibody. J. Immunol. 97: 840-853.

Jacob, F and Monod, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318-356.

Jameson, E. 1937. A phase rule study of blood serum: The effect of changes in certain variables. J. Gen. Physiol. 20: 859-877.

Janeway, C.A. and Citlin, D. 1957. The gama globulins. Advan. Pediat. 9: 65-136.

Jeghers, H. and Selesnick, S. 1937. Hyperproteinemia: Its significance. Internat. Clin. 3: 248-279.

Jerne, N.K. 1955. The natural selection theory of antibody formation. Proc. Natl. Acad. Sci. U.S. 41: 849.

Kabat, E.A. 1957. Size and heterogeneity of the combining sites on an antibody molecule. J. Cellular Comp. Physiol. (Suppl.) 1: 79-102.

Karusch, F. 1957. The role of disulfide bonds in antibody specificity. J. Am. Chem. Soc. 79: 5323.

Karusch, F. 1962. Immunologic specificity and molecular structure. Advan. Immunol. 2: 1-39.

Kekwick, R.A. and Record, B.R. 1941. Some physical properties of diptheria antitoxic horse sera. Brit. J. Exp. Pathol. 22: 29-44.

Korngold, L. and Lipari, R. 1956. Multiple myeloma proteins. III. The antigenic relationships of Bence Jones proteins to normal gamma globulin and multiple myeloma serum proteins. Cancer 9: 262-272.

Koshland, M.E. and Englberger, F.M. 1963. Differences in the Amino acid composition of two purified antibodies from the same rabbit. Proc. Natl. Acad. Sci. U.S. 50: 61-68.

Kunkel, H.G., Allen, J.C., and Grey, H.M. 1964a. Genetic characters and the polypeptide chains of various types of gamma globulin. Cold Spring Harbor Symp. Quant. Biol. 29: 443-447.

Kunkel, H.G., Allen, J.C., Grey, H.M., Martensson, L., and Grubb, R. 1964b. A relationship between the H-chain groups of 7S gamma globulin and the Gm system. Nature 203: 413-414.

Kunkel, H.G., Grey, H.M., and Soloman, A. 1965. Proc. IVth Intern. Congr. Immunopathol. Cited by Kunkel, H.G., Kellander, J. and Mannik, M. 1966. Current trends in immune globulin research. Acta Med. Scand. (Suppl.) 445: 64.

Kunkel, H.G., Kellander, J., and Mannik, M. 1966. Current trends in immune globulin research. Acta Med. Scand. (Suppl.) 445: 63-73.

Kunkel, H.G., Mannik, M., and Williams, R.C. 1963. Individual antigenic specificity of isolated antibodies. Science 140: 1218-1219.

Kunkel, H.G. and Slater, R.J. 1952. Zone electrophoresis in a starch supporting medium. Proc. Soc. Exp. Biol. Med. 80: 42-44.

Lamm, M.E., Nuzzenzweig, V. and Benaceraf, B. 1966. Isolation of purified H- and L-polypeptide chains from guinea-pig immunoglobulin after mild reduction. Immunology 10: 309-319.

Larson, D.L. and Tomlinson, L.J. 1952. Quantitative antibody studies in man. J. Lab. Clin. Med. 39: 129-134.

Laurell, C.-B. and Heremans, J.F. 1961. Comparison between electrophoretic patterns and hexose content of different immunological types of M-components. In studies on "abnormal" serum globulins (M-components) in myeloma, macroglobulinemia and related diseases. Acta Med. Scand. (Suppl.) 367: 101-109.

Lawler, S.D. and Cohen, S. 1965. Distribution of allotypic specificities on peptide chains of human gamma globulin. Immunology 8: 206-212.

Lawson, H.A., Stuard, C.A., Paull, A.M., Phillips, A.M., and Phillips, R.W. 1955. Observations on antibody content of blood in patients with multiple myeloma. New Engl. J. Med. 252: 13-18.

Lederberg, J. 1959. Genes and antibodies. Science 129: 1649-1653.

Levin, W.C. and Ritzmann, S.E. 1966. Relation of abnormal proteins to formed elements of blood: Effects upon erythrocytes, leukocytes, and platelets. Ann. Rev. Med. 17: 323-326.

Lieberman, R., Dray, S. and potter, M. 1965. Linkage in control of allotypic specificities on two different γG-immunoglobulins. Science 148: 640-642.

Magnus-Levy, A. 1936. Ueber krystallisiertes and amorphes Bence Jones eiwess. Z. Physiol. Chem. 243: 173-188.

Manner, G., Gould, B.S. and Slayter, H.S. 1965. Gamma globulin synthesis on ribosomes and ribosomal aggregates. Biochim. Biophys. Acta 108: 659-676.

Mannik, M. and Kunkel, H.G. 1962. Classification of myeloma proteins, Bence Jones proteins and macroglobulins into two groups on the basis of common antigenic characters. J. Exp. Med. 116: 859-877.

Mannik, M. and Kunkel, H.G. 1963a. Antigenic specificity of individual "monoclonal γ-globulins". Federation Proc. 22: 264.

Mannik, M. and Kunkel, H.G. 1963b. Two major types of normal 7S γ-globulin. J. Exp. Med. 117: 213-230.

Mannik, M. and Kunkel, H.G. 1965. The immunoglobulins. In: Samter, M. (ed.) Immunological Diseases. Little, Brown and Co., Boston. pp. 278-304.

Marcus. 1899. Z. Physiol. Chem. 28: 559. Cited by Hardy, W.B. 1905. Colloidal solution: The globulins. J. Physiol. 33: 333.

Marler, E., Nelson, C.A. and Tanford, C. 1964. The polypeptide chains of rabbit gamma globulin and its papain-cleaved fragments. Biochemistry 3: 279-284.

Marmont, A.M., Fusco, F.A., Gay, A. and Mariotti, L. 1957. Intravascular erythrocyte aggregation ("sludged blood") in multiple myeloma. Acta Haemat. 18: 49-58.

Martensson, L. and Kunkel, H.G. 1964. H-chain subgroups of myeloma proteins and normal 7S γ-globulin. J. Exp. Med. 120: 253-266.

Martensson, L. and Kunkel, H.G. 1965. Distribution among the γ-globulin molecules of different antigenic specificities in the Gm system. J. Exp. Med. 122: 799-811.

Mellors, R.C. and Korngold, L. 1963. The cellular origin of human immunoglobulins (γ_2, γ_{1M}, γ_{1A}). J. Exp. Med. 118: 387-396.

Merler, E. and Rosen, F.S. 1966. The gamma globulins. I. The structure and synthesis of the immunoglobulins. New Engl. J. Med. 275: 480-486.

Meyer, F. 1962. In vitro incorporation of C^{14}-lysine into Bence Jones protein. Proc. Soc. Exp. Biol. Med. 110: 106-109.

Meyer, F., Putnam, F.W. 1963. The fate of injected Bence Jones protein. J. Exp. Med. 117: 573-581.

Migita, S. and Putnam, F.W. 1963. Antigenic relationships of Bence Jones proteins, myeloma globulins, and normal human γ-globulin. J. Exp. Med. 117: 81-104.

Milstein, C. 1964. Disulphide bridges and dimers of Bence Jones protein. J. Mol. Biol. 9: 836-839.

Milstein, C. 1966. Variations in amino-acid sequence near the disulphide bridges of Bence Jones protein. Nature 209: 370-373.

Molo, F., Governa, M., Citati, C. and Dellepiane, M. 1963. Experimental observations on the participation of macrophages in the mechanism of antibody production. Experientia 19: 582-583.

Nachman, R.L., Engel, R.L., Jr., and Stein, S. 1965. Subtypes of Bence Jones proteins. J. Immunol. 95: 295-299.

Naumann, H.N. 1965. Differentiation of Bence Jones protein from uroglobulins. Am. J. Clin. Pathol. 44: 413-425.

Nisonoff, A.G., Marcus, G., and Wissler, F.C. 1961. Separation of univalent fragments of rabbit antibody by reduction of single labile disulphide bond. Nature 189: 293-295.

Nisonoff, A. and Rivers, M.M. 1961. Recombination of a mixture of univalent antibody fragments of different specificities. Arch. Biochem. Biophys. 93: 460-462.

Nisonoff, A., Wissler, F.C., Lipman, L.N., and Woernley, D.L. 1960. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. Arch. Biochem. Biophys. 89: 230-244.

Noelken, M.E., Nelson, C.A., Buckely, C.E., and Tanford, C. 1965. Gross conformation of rabbit 7S γ-immunoglobulin and its papain-cleaved fragments. J. Biol. Chem. 240: 218-224.

Noelken, M.E. and Tanford, C. 1964. Unfolding and renaturation of a univalent antihapten antibody fragment. J. Biol. Chem. 239: 1828-1832.

Norgaard, O. 1964. Recherches sur l'evolution preclinique due myelome multiple. Acta Med. Scand. 176: 137-146.

Nossal, G. J., Ada, G.L., and Austin, C.M. 1964. Antigens in immunity II. Immunogenic properties of flagella, polymerized flagellin and flagellin in the primary response. Aust. J. Exp. Biol. Med. Sci. 42: 282-294.

Nossal, G.J., Ada, G.L., and Austin, C.M. 1965. IX. The antigen content of single antibody-forming cells. J. Exp. Med. 121: 945-954.

Nussenzweig, F., Franklin, E.C. and Benacerraf, B. 1964. Immunologic properties of products obtained by mild reduction of human and guinea pig γ-globulins. J. Immunol. 93: 1015-1023.

Ogruyzlo, M.A., Maclachlan, M., Dauphinee, J.A. and Fletcher, A.A. 1959. Serum proteins in health and disease: Filter paper electrophoresis. Am. J. Med. 27: 596-616.

Olins, D.E. and Edelman, G.M. 1962. The antigenic structure of the poly-peptide chains of human γ-globulin. J. Exp. Med. 116: 635-651.

Olins, D.E. and Edelman, G.M. 1964. Reconstitution of 7S molecules from L and H polypeptide chains of antibodies and γ-globulins. J. Exp. Med. 119: 789-815.

Osserman, E.F. 1965. Multiple myeloma. In: Sampter, M. (ed.) Immunological Diseases. Little, Brown and Co., Boston. pp. 353-363.

Osserman, E.F. and Lawlor, D.P. 1961. Immunoelectrophoretic characterization of the serum and urinary proteins in plasma cell myeloma and Waldenstrom's macroglobulinemia. Ann. N.Y. Acad. Sci. 94: 93-109.

Osserman, E.F. and Takatsuki, K. 1963. Plasma cell myeloma: Gamma globulin synthesis and structure. Medicine 42: 357-384.

Osserman, E.F. and Takatsuki, K. 1964. Clinical and immunochemical studies of four cases of heavy ($H^{\gamma 2}$) chain disease. Am. J. Med. 37: 351-373.

Oudin, J. 1946. Method of immunochemical analysis by specific precipitation in gelled medium. C. R. Acad. Sci. (Paris) 222: 115-116.

Palmer, J.L., Mandy, W.J., and Nisonoff, A. 1962. Heterogenity of rabbit antibody and its subunits. Proc. Natl. Acad. Sci. U.S. 48: 49-53.

Pernis, B. and Chiappino. 1964. Identification in human lymphoid tissues of cells that produce group 1 or group 2 gamma globulins. Immunology 7: 500-506.

Pohl, F. 1886. Arch. Exp. Pathol. Pharmakol. 20: 426. Cited by Haurowitz, F. 1963. The Chemistry and Function of Proteins. Academic Press, New York. p. 184.

Pope, C.G. 1939. Action of proteolytic enzymes on antitoxins and proteins in immune sera: True digestion of proteins. Brit. J. Exp. Pathol. 20: 132-201.

Porges and Spiro. 1902. Beit. Z. Physiol. Pathol. 3: 277. Cited by Hardy, W.B. 1905. Colloidal solution: The globulins. J. Physiol. 33: 333.

Porter, R.R. 1950. A chemical study of rabbit antivalbumin. Biochem. J. 46: 473-478.

Porter, R.R. 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. Biochem. J. 73: 119-126.

Porter, R.R. 1960. Gamma globulin and antibodies. In: Putnam, F.W. (ed.). The Plasma Proteins. Academic Press, New York. Vol. 1. pp. 241-277.

Porter, R.R. 1962. Structure of gamma-globulin and antibodies. In: Gellhorn, A. and Hirschberg, E. (eds.) Basic Problems In Neoplastic Disease. Columbia Univ. Press, New York. pp. 177-191.

Potter, M., Apella, E., and Geisser, S. 1965. Variations in the heavy polypeptide chain structure of gamma myeloma immunoglobulins from an inbred strain of mice and a hypothesis as to their origin. J. Mol. Biol. 14: 361-372.

Putnam, F.W. 1955. Abnormal human serum globulins. Science 122: 275-277.

Putnam, F.W. 1962. Structural relationships among normal human γ-globulin myeloma globulins, and Bence Jones proteins. Biochim. Biophys. Acta 63: 539-541.

Putnam, F.W., Easley, C.W., Lynn, L.T., Ritchie, A.E. and Phelps, R.A. 1959. The heat precipitation of Bence Jones proteins. I. Optimum conditions. Arch. Biochem. 83: 115-130.

Putnam, F.W. and Easley, C.W. 1965. Structural studies of the immunoglobulins. I. The tryptic peptides of Bence Jones proteins. J. Biol. Chem. 240: 1626-1638.

Ramel, A., Stellwagen, E. and Schachman, H.K. 1961. Subunits of proteins. Federation Proc. 20: 387.

Rawson, A.J. and Sunderman, F.W. 1948. Studies in serum electrolytes: XV. The calcium binding property of serum proteins (multiple myeloma, lymphogranuloma, venereum and sarcoidosis). J. Clin. Invest. 27: 82-90.

Remington, J.S., Merler, E., Lerner, A.M., Gitlin, D. and Finland, M. 1962. Antibodies of low molecular weight in normal human urine. Nature 194: 407-408.

Riva, G. 1957. Das Serumeiweissbild. Hans Huber. Bern. p. 631.

Rogentine, N.G., Rowe, D.S., Bradley, J., Waldman, T.A. and Fahey, J.L. 1966. Metabolism of human immunoglobulin D (IgD). J. Clin. Invest. 45: 1467-1478.

Rowe, D.S. and Fahey, J.L. 1965a. New class of human immunoglobulins. I. A unique myeloma protein. J. Exp. Med. 121: 171-184.

Rowe, D.S. and Fahey, J.L. 1965b. New class of human immunoglobulins. II. Normal serum IgD. J. Exp. Med. 121: 185-199.

Sankuhler, S. 1952. A simple and sensitive test for Bence Jones protein. Am. J. Clin. Pathol. 22: 282-284.

Scharff, M.D. and Uhr, J.W. 1965. Functional ribosomal unit of gammaglobulin synthesis. Science 148: 646-648.

Scheidegger, J.J. 1955. Une micro-methode de L'immunoelectrophorese. Intern. Arch. Allergy Appl. Immunol. 7: 103-110.

Schlossman, S.F. and Kabat, E.A. 1962. Specific fractionation of population of antidextran molecules with combining sites of various sizes. J. Exp. Med. 116: 535-552.

Seligmann, M. 1965. Proc. IVth Intern. Congr. Immunopathol. Cited by Kunkel, H.G., Kellander, J. and Mannik, M. 1966. Current trends in immune globulin research. Acta Med. Scand. (Suppl.) 445: 66.

Seligmann, M., Mihoesco, C. and Meshaka, G. 1966. Antigenic determinants common to human immunoglobulins G and M: Importance of conformational antigens. Science 154: 790-791.

Shapiro, A.L., Scharff, M.D., Maizel, J.V. and Uhr, J.W. 1966. Synthesis of excess light chains of gamma globulin by rabbit lymph node cells. Nature 211: 243-245.

Singer, S.J. 1965. Structure and function of antigen and antibody proteins. In: Neurath, H. (ed.). The Proteins. Academic Press, Inc. New York. Vol. 3: 269-357.

Singer, S.J. and Doolittle, R.F. 1966. Antibody active sites and immunoglobulin molecules. Science 153: 13-25.

Skvaril, F. 1960. Changes in outdated human γ-globulin preparations. Nature 185: 475-476.

Slater, R.J., Ward, M.S. and Kunkel, H.G. 1955. Immunological relationships among the myeloma proteins. J. Exp. Med. 101: 85-108.

Smith, J.K. 1963. The significance of the "protein error" of indicators in the diagnosis of Bence Jones proteinuria. Acta Haemat. 30: 144-152.

Smithies, O. 1963. Gamma globulin variability: A genetic hypothesis. Nature 199: 1231-1236.

Solomon, A. and Fahey, J.L. 1964. Bence Jones proteinuria. Am. J. Med. 37: 206-222.

Solomon, A., Fahey, J.L. and Malmgren, R.A. 1963a. Immunohistologic localization of gamma-1-macroglobulins, beta-2A myeloma proteins, 6.6S gamma-myeloma proteins, and Bence Jones proteins. Blood 21: 403-423.

Solomon, A., Litwin, S.D. and Kunkel, H.G. 1965. Marked antigenic and other differences among Bence Jones proteins containing similar genetic factors. Federation Proc. 24: 201.

Solomon, A., Waldmann, T.A. and Fahey, J.L. 1963b. Metabolism of normal 6.6S γ-globulin in normal subjects and in patients with macroglobulinemia and multiple myeloma. J. Lab. Clin. Med. 62: 1-17.

Solomon, A., Waldman, T.A., Fahey, J.L. and MacFarlane, A.S. 1964. Metabolism of Bence Jones proteins. J. Clin. Invest. 43: 103-117.

Stein, S., Nachman, R.L. and Engle, R.L., Jr. 1963. Individual and sub-group antigenic specificity of Bence Jones protein. Nature 200: 1180-1181.

Stenzel, K.H., Phillips, W.D., Thompson, D.D. and Rubin, A.L. 1964. Functional ribosomes in antibody-producing cells. Proc. Natl. Acad. Sci. U.S. 51: 636-642.

Stevenson, G.T. 1960. Detection in normal urine of protein resembling Bence Jones protein. J. Clin. Invest. 39: 1192-1200.

Stiehm, E.R., Morton, J.I. and Deutsch, H.F. 1960. Some immunologic properties of papain digest fragments of multiple myeloma proteins. J. Immunol. 85: 337-341.

Svensson, H. 1941. Fractionation of serum with ammonium sulfate water dialysis, studied by electrophoresis. J. Biol. Chem. 139: 805-825.

Talmage, D.W. and Cohen, E.P. 1965. Antibody production and specificity. In: Samter, M. (ed.). Immunological Diseases. Little, Brown and Co. Boston. pp. 87-99.

Talmage, D.W. and Cohen, E.P. 1965. Antibody production and immune responses. In: Samter, M. (ed.). Immunological Diseases. Little, Brown and Co. Boston. pp. 109-121.

Tawde, S., Scharff, M.D. and Uhr, J.W. 1966. Mechanisms of γ-globulin synthesis. J. Immunol. 96: 1-7.

Terry, W.D. and Fahey, J.L. 1964. Subclasses of human γ_2 globulin based on differences in heavy polypeptide chains. Science 146: 400-401.

Tiselius, A. 1930. Nova Acta Soc. Sci. Upsalien. 4: 7. Cited by Tiselius, A. 1937a. Electrophoresis of serum globulin. Biochem. J. 31: 313.

Tiselius, A. 1937a. Electrophoresis of serum globulin. Biochem. J. 31: 313-317.

Tiselius, A. 1937b. Electrophoresis of serum globulin. II. Electrophoretic analysis of normal and immune sera. Biochem. J. 31: 1464-1477.

Tiselius, A. 1937c. Electrophoresis of purified antibody preparations. J. Exp. Med. 65: 641-646.

Tiselius, A. and Eriksson-Quensel, I.B. 1939. CCXVIII. Some observations on peptic digestion of egg albumin. Biochem. J. 33: 1752-1756.

Titani, K. and Putnam, F.W. 1965. Immunoglobulin structure: Amino-and carboxyl-terminal peptides of type I Bence Jones proteins. Science 147: 1304-1305.

Titani, K., Whitley, E., Avogardo, L. and Putnam, F.W. 1965. Immunoglobulin structure: Partial amino acid sequence of a Bence Jones protein. Science 149: 1090-1092.

Titani, K., Whitley, E., Jr. and Putnam, F.W. 1966. Immunoglobulin structure: Variation in the sequence of Bence Jones proteins. Science 152: 1513-1515.

Titani, K., Wikler, M. and Putnam, F.W. 1967. Evolution of immunoglobulins: Structural homology of kappa and lambda Bence Jones proteins. Science 155: 828-835.

van der Scheer, J., Wycoff, R.W.G. and Clarke, F.H. 1940. The electrophoretic analysis of several hyperimmune horse sera. J. Immunol. 39: 65-71.

van Furth, R., Schuit, H.R. and Hijamans, W. 1964. De vorming von immunoglobulinen door menselijke weefsels in vitro. Nederl T. Geneesk 108: 2339-2340.

Watson, J.D. 1965. Molecular Biology of the Gene. W.A. Benjamin, Inc. New York. p. 177

Webb, T., Rose, B. and Sehon, A.H. 1958. Biocolloids in normal urine: II. Physiochemical and immunochemical characteristics. Can. J. Biochem. Physiol. 36: 1167.

Wellensiek, H.J. and Coons, A.H. 1964. Studies on antibody production. IV. The cellular localization of antigen molecules (ferritin) in the secondary response. J. Exp. Med. 119: 685-696.

Whitney, P.L. and Tanford, C. 1965. Recovery of specific activity after complete unfolding and reduction of an antibody fragment. Proc. Natl. Acad. Sci. U.S. 53: 524-532.

Williams, C.A. and Grabar, P. 1955. Immunoelectrophoretic studies on serum proteins: I. The antigens of human serum. J. Immunol. 74: 158.

Williams, R.C., Jr. 1964. Heterogenicity of L-chain sites on Bence Jones proteins reacting with anti-gamma globulin factors. Proc. Natl. Acad. Sci. U.S. 52: 60-64.

Williams, R.C., Brunning, R.D. and Wollheim, F.A. 1966. Light-chain disease. Ann. Intern. Med. 65: 471-486.

World Health Organization. 1964. Nomenclature for human immunoglobulins. Bull. World Health Organ. 30: 447-450.

World Health Organization. 1965. Notation for genetic factors of human immunoglobulins. Bull. World Health Organ. 33: 721-724.

Wuhrman, F. and Marki, H.H. 1963. Dysproteinamien und paraproteinamein; Grundlagen, klinik and therapie. Schwabe and Co. Basel/Stuttgart.

VITA

Name	Lora Allred
Birthplace	Lovell, Wyoming
Birthdate	17 May 1937
Elementary School	Lovell Elementary Lovell, Wyoming Sherman Elementary Salt Lake City, Utah Roosevelt Elementary Salt Lake City, Utah
High School	Granite High School Salt Lake City, Utah
University	University of Utah Salt Lake City, Utah 1955-1960
Degree	B.S., University of Utah Salt Lake City, Utah, 1960
Licensure and Registration	American Society of Clinical Pathologists California Department of Public Health
Professional Organizations	American Society of Medical Technologists Utah Society of Medical Technologists
Professional Positions	Department of Medicine, University of Utah; Medical Technologist 1957-1959, 1961-1963; Department of Pathology, Salt Lake County General Hospital; Medical Technologist, 1959-1960, 1963; Cancer Research Institute, University of California Medical Center; Medical Technologist, 1960-1961; Research Division, Carney Hospital; Medical Technologist, 1961; Department of Pathology, Latter-Day Saints Hospital; Medical Technologist, 1963-1966; Department of Microbiology, University

Professional Positions, cont.	of Utah; Teaching Assistant, 1965; School of Medical Technology, University Hospital; Teaching Supervisor, 1966-; Summer Teaching Institute, University of Minnesota, Participant, 1966; Administrative Committee on Medical Technology, University of Utah; Member 1966-; Board of General Education Counselors, University of Utah; Member 1967-.
Publications	Hunter, D.T., Jr., Allred, L. and Miller, J.C. (in press). Concentration of cerebrospinal fluid by ultra filtration. Amer. J. Med. Tech.